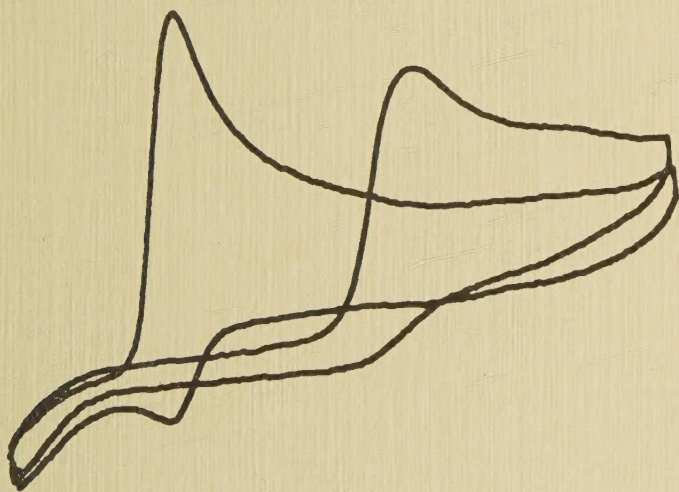


*A Study of the Electrochemical
Oxidation of Reduced Coenzymes
at Modified Electrodes*



Lund 1982

Anne Tontum

A. Torstenssen



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A STUDY OF THE ELECTROCHEMICAL OXIDATION OF REDUCED
NICOTINAMIDE COENZYMES AT MODIFIED ELECTRODES

by

Arne Torstensson, fil.kand., Sm



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Abstract Coulometric determination of <u>1,4-dihydronicotinamide adenine dinucleotide (NADH)</u> at a rotating platinum gauze electrode at + 0.7 V vs SCE is described. The current efficiency for the oxidation is at least 99.3%. The charge transfer interaction between the nicotinamide and the adenine part in the coenzyme in solution, determined by UV-spectroscopy was found to be very small. A <u>coenzyme-enzyme complex</u> has been covalently coupled to a <u>glassy carbon electrode</u>. The attached coenzyme could be oxidized only once on cyclic voltammetric experiments. <u>Chemical modifications of graphite electrodes</u> by adsorption of mediators at the electrode surface have been successful in decreasing the high overvoltage of the <u>coenzyme oxidation</u>. The mediators used for the electrocatalysis were <u>alkylphenazinium ions</u>, <u>catechols</u>, and a <u>phenoxazinium ion</u>. The electron transfer between the attached mediators and the electrodes was fast.		
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A STUDY OF THE ELECTROCHEMICAL OXIDATION OF REDUCED NICOTIN-
AMIDE COENZYMES AT MODIFIED ELECTRODES

This thesis summarizes the work presented in the following
papers, referred to in the discussion by the Roman numerals I-VI

- I Electrochemical Oxidation of Reduced Nicotinamide Adenine
 Dinucleotide Directly and After Reduction in an Enzyme
 Reactor
 Jaegfeldt, H.; Torstensson, A.; Johansson, G.
 Anal. Chim. Acta 97(1978)221-228
- II Spectroscopic and Theoretical Studies on the Intramolecular
 Electron Transfer in NAD
 Mitra, C.; Torstensson, A.
 Bioelectr. Bioenerg. 5(1978)601-606
- III Electrochemical Regeneration of NAD Covalently Bound to
 Liver Alcohol Dehydrogenase
 Torstensson, A.; Johansson, G.; Månsson, M.O.; Larsson, P.O.;
 Mosbach, K.
 Anal. Lett. 13(1980)837-849
- IV Catalytic Oxidation of NADH by Surface-Modified Graphite
 Electrodes
 Torstensson, A.; Gorton, L.
 J. Electroanal. Chem. 130(1981)199-207



V Catalytic Oxidation of Reduced Nicotinamide Adenine
Dinucleotide by Graphite Electrodes Modified with Adsorbed
Aromatics Containing Catechol Functionalities
Jaegfeldt, H.; Torstensson, A.B.C.; Gorton, L.G.O.;
Johansson, G.
Anal. Chem. 53(1981)1979-1982

VI Catalytic Oxidation of Reduced Nicotinamide Adenine
Dinucleotide by Graphite Electrodes Modified with
Adsorbed Phenoxazinium Salts
Gorton, L.G.O.; Torstensson, A.B.C.; Johansson, G.
submitted to Anal. Chem.

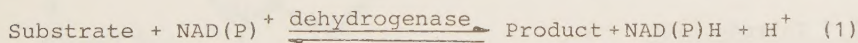
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1. INTRODUCTION

In 1905, Harden and Young [1] could separate Buchner's "zymase" into a high and a low (coenzyme) molecular weight component. The coenzyme (Fig. 1) was isolated 25 years later by Euler and co-workers [2]. It was then known by several names codehydrogenase I, coenzyme I and later on DPN (diphosphopyridine nucleotide) and it is presently named NAD^+ (nicotinamide adenine dinucleotide). Warburg and Christian [3] discovered the closely related NADP^+ (nicotinamide adenine dinucleotide phosphate).

In the living cell there are many enzyme systems that require either NAD^+ or NADP^+ for their activity. Those enzymes are called dehydrogenases and hitherto more than 400 of them have been isolated [4]. Their common biochemical reaction scheme is



The coenzyme serves as an electron carrier in the redox reaction. The reduced coenzyme is used either for synthetic purposes (NADPH) or for hydrogen transfer through the respiratory chain (NADH).

It has not yet been possible to obtain a reversible electron transfer to an electrode surface. In the middle of the 50:s Rodkey [5] started potentiometric measurements on the NAD^+/NADH redox system using benzylviologen as mediator. He found that an additional enzyme system was required to obtain equilibrium between the solution and the electrode. Since then, the

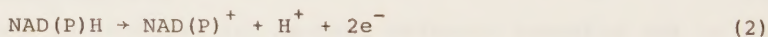
the electrochemical properties of $\text{NAD(P)}^+/\text{NAD(P)H}$ system have been studied rather extensively [6-13]. Tse and Kuwana [14] have shown the feasibility of fabricating chemically modified electrodes capable of catalysing the electrochemical oxidation of NADH. They introduced an electroactive mediator at the electrode which decreased the high overvoltage by some hundred millivolt.

Better knowledge about the electrochemical properties of the redox coenzyme couples, their interactions with mediators and further development of chemically modified electrodes is indispensable for approaching a reversible behaviour. The co-enzyme/modified electrode system could then be advantageously used for analytical purposes, in bio-fuel cells and so forth.

2. ELECTROCHEMISTRY OF NAD(P)H

2.1 Introduction

The electrochemical oxidation of NAD(P)H is an overall 1 H^+ , 2 e^- process (eq 2)



An ECE mechanism is proposed as the main reaction at Pt [13] and at carbon electrodes [12]. The removal of the first electron is the rate-limiting step and it causes the high overvoltage. The oxidation peak potential found by cyclic voltammetry is about +600 mV vs SCE for Pt and around +400 mV vs SCE for carbon electrodes. The reversible redox-potential at pH 7.0 is -560 mV vs SCE [5] which means that the overvoltage is more than 1.1 V for Pt and more than 0.9 V for carbon electrodes.

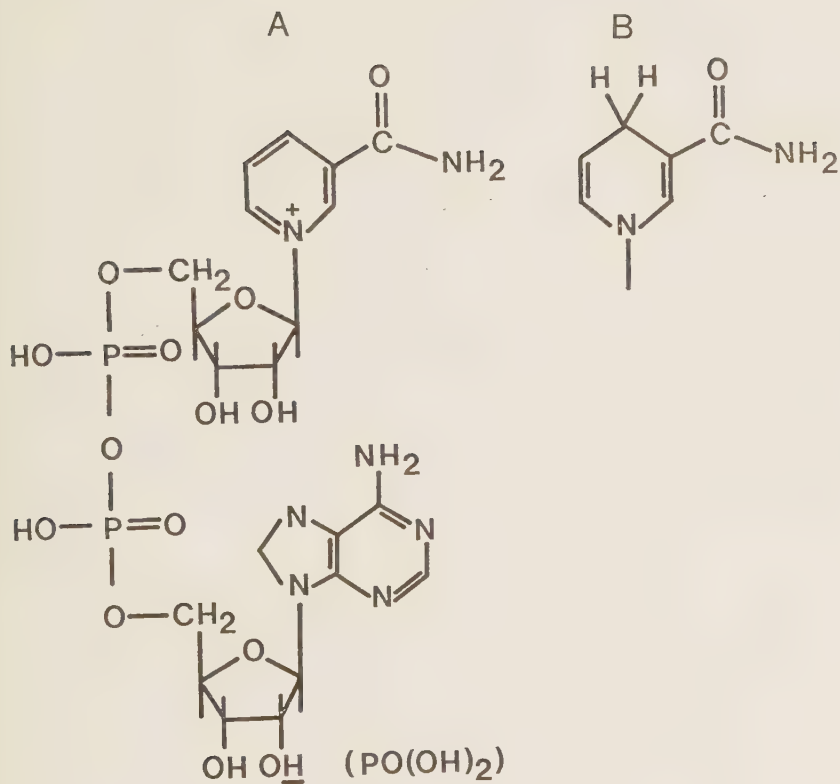


Fig. 1. The structure of NAD(P)⁺ (A) and NAD(P)H (B)

According to eq 2 the electrochemical reaction is pH-dependent and a decrease of 30 mV/pH unit is expected. Only a slight pH-dependence (~ 15 mV/pH unit) has been reported [10] between pH 7-10. Blaedel and Jenkins have shown that the ease of oxidation of NADH depends upon the nature of buffer. Tris-HCl was found to have an inhibitory effect. Another problem which arises when oxidizing NAD(P)H electrochemically is that the electrode surface gets fouled. The peak will shift towards more positive potential on successive cyclic scans and a reproducible cyclic voltammogram is obtained only after 5 to 10 scans. This phenomenon can probably be attributed to the adsorption of NAD(P)^+ which then passivates the electrode towards the oxidation of NAD(P)H. Preadsorption of NAD^+ on the electrode surface has been suggested as a method to obtain reproducible cyclic voltammograms at glassy carbon electrodes [11].

The variation in peak potential for the oxidation of NAD(P)H between different states of electrode surfaces seems to have very much to do with surface oxides. The electron transfer between the coenzyme and the electrode probably takes place through these oxygen functionalities at carbon electrodes.

2.2 Electrode pretreatment

The state of the electrode surface is of outmost importance when investigating the behaviour of NAD(P)H. Pretreated electrodes are therefore necessary to increase the active surface and to obtain precise and reproducible results (paper I). An improved method for pretreatment is given by Jaegfeldt [13].

The Pt-electrodes are dipped in hot concentrated sulphuric acid, rinsed with water and repeatedly subjected to a potential scan between -850 mV and +1000 mV vs SCE in a buffer solution until the characteristic adsorption peaks for hydrogen are obtained. For carbon electrodes Blaedel and Jenkins [10] have proposed another pretreatment procedure. They applied a potential of +1.35 V for two minutes and then switched to -1.35 V for another two minutes, in deaerated buffer.

2.3 Coulometric analysis of NAD(P)H

In controlled potential coulometry the total number of coulombs consumed in the electrolysis is used to determine the amount of electrolyzed substance. A three-electrode potentiostat with electronic integration and direct read-out of the current-time integral was used. The current-time course follows (eq 3)

$$i(t) = i_0 \cdot e^{-p \cdot t} \quad (3)$$

where i_0 = initial current, p = constant, depending on the experimental conditions.

The total quantity of electricity $Q(t)$ is given by the area under the i - t curve and can be calculated from (eq 4)

$$Q(t) = \int_0^t i(t) dt \quad (4)$$

Faraday's law (eq 5) is the basis for the coulometric method of analysis,

$$Q(t) = n \cdot F \cdot X \quad (5)$$

where X represents the total number of moles of substance initially present.

Earlier studies of the oxidation of NAD(P)H reported a coulometric n-value (eq 5) less than two on large-scale controlled electrode potential electrolysis [7,15]. For coulometric measurements of NAD(P)H only Pt-electrodes have been used so far. With an appropriate experimental set up and a pretreated electrode a coulometric determination of the amount of NADH present in solution is readily performed (paper I). The results agree very well with data obtained spectrophotometrically. This method has a limited application in analysis due to the high potential applied to the working electrode in order to bring about the oxidation. It is very important to note that almost 100% current efficiency could be obtained when oxidizing the coenzyme electrochemically.

2.4 Oxidation specificity

In a coenzyme regeneration procedure it is important to obtain the desired product in a high yield. If inactive side products are generated, the reaction will slow down after few cycles due to a decrease in the concentration of the coenzyme (table I).

Table I. Number of cycles which reduces the coenzyme content to 50% due to the relative specificity of the oxidation reaction.

Rel. specificity/%	Number of cycles
90	7
95	14
99	69
99.3	99
99.9	690

The oxidation regeneration procedure was studied in paper I for NADH at a Pt-electrode. The reverse reaction was brought about by alcoholdehydrogenase (ADH) immobilized on porous glass in an enzyme reactor. It was found that the reaction specificity was at least 99.3%, when the chemical stability of the coenzyme was taken into account.

An electrochemical NAD(P)^+ -regeneration method can be a good alternative to chemical oxidizing reagents or enzymatic methods.

2.5 Coenzyme analogues

Much attention has been paid to modifications of the coenzyme in the adenine part. The purposes have been to bind the coenzyme to an electrode [16], to a column [17] or even to an enzyme [18]. Such coenzyme derivatives have retained its activity in enzymatic reactions. The modification does not interfere with the electrochemical oxidation of the analogue, since the redox reaction occurs at the nicotinamide part of the molecule (paper III). Attached to an electrode surface via a coupling to the adenine part, the redox site is separated from the electrode material. The electron transfer can then either occur via a flip-flop movement, electron tunneling or intramolecular charge transfer. There is, however, practically no intramolecular charge transfer interaction between the nicotinamide and the adenine part in solution (paper II). The backbone of the coenzyme is also electronically insulating. It is therefore impossible to exploit the adenine part in a coupling reaction to an electrode surface in order to facilitate the electron transfer. Modification in the nicotinamide part is prohibited from enzymatic point of view.

CHEMICALLY MODIFIED ELECTRODES

3.1 Introduction

As mentioned earlier adsorption on an electrode surface can cause severe difficulties in the investigations of electrochemical reactions. It can interfere with the electron transfer process and it can make the evaluation uncertain. Irreversible adsorption for a particular redox couple can, however, be very useful. With covalent coupling or polymer coating of electroactive molecules to an electrode surface a new interesting area was opened, leading to the development of chemically modified electrodes (CME:s) [19]. One of the first reports on a CME was the "chiral electrode" [20]. In electrosynthesis this electrode gave an optical active product. Up to now there have been a considerable number of papers dealing with this subject. Table II gives some examples of various types of CME:s.

The main contributions of CME:s are in the fields of:

- 1) study of intramolecular and electrochemical transfer
- 2) electrocatalysis; where the attached redox couple plays the role of an electron carrier between the substrate and the electrode
- 3) electrosynthesis

3.2 Preparation methods

Covalent coupling

For the covalent coupling of the species of interest a number of methods based on rather simple organic reactions are available. For carbon electrodes the introduction of oxygen-functionalities at the surface is often the first step for

Table II

Some examples of various types of chemically modified electrodes (CME:s).

Electrode material	Immobilization procedure	Coupling reagent	Redox system	References
SnO ₂	covalent coupling	organosilanes	-	(21)
- "- -	chemisorption*	-	iron porphyrin	(22)
RuO ₂	covalent coupling	organosilanes	nitrobenzamides	(23)
- "- -	- " -	- " -	tetrathiafulvene	(24)
TiO ₂	- " -	- " -	alkylpyridinium ion	(25)
Pt/PtO	chemisorption	allylamine		(26)
- "- -	covalent coupling	organosilanes	3,5 dinitrobenzamide	(27)
- "- -	chemisorption	-	vinylferrocene	(28)
- "- -	covalent coupling	allylamin	ferrocene carbaldehyd	(29)
- "- -	polymer coating	-	tetrathiafulvene	(30)
glassy carbon	covalent coupling	organosilane	-	(31)
- "- -	- " -	thionyl chloride	tetra (p-aminophenyl) porphyrin	(32)
- "- -	- " -	cyanuric chloride	3,4-dihydroxybenzyl-amine	(14)
- "- -	- " -	cyanuric chloride	coenzyme-enzyme complex	paper III
graphite	adsorption	-	9,10-phenanthro-quinone	(33)
- "- -	- " -	1-(9-phenanthrene)-2-(4-pyridine)-ethene	ruthenium complex	(34)
- "- -	covalent coupling	dicyclohexyl-carbodiimide	- " -	(35)
- "- -	polymer coating	-	- " -	(36)
- "- -	- " -		amino ferrocenes	(37)
- "- -	adsorption	-	metallo porphyrins	(38)
- "- -	covalent coupling	cyanuric chloride	glucose-oxidase	(39)
- "- -	adsorption	-	NAD(P)H-mediators	paper IV-VI
carbon paste	covalent coupling	n-octaldehyd	NAD ⁺	(16)
- "- -	adsorption	-	1,2 naphtoquinone	(40)
carbon fibre	covalent coupling	organosilane		(41)

possible coupling reactions. This can be performed in various ways e.g. thermal, O_2 plasma, or chemical oxidation. The molecule to be coupled must have a peripheral functionality for the attachment. In this case it is often an NH_2 -group, which then yields an amide linkage with an activated carboxylic group at the electrode. The covalent coupling can also be formed by using an intermediate bifunctional molecule, as alkyl-silanes or cyanuric chloride, which will act as a linkage between the electrode and the redox couple. In a good covalent coupling method the attachment must be

- 1) stable in the potential region of interest.
- 2) chemically stable in the solution.
- 3) electrochemically inert.

The combination of O_2 plasma oxidation and cyanuric chloride invented by Kuwana [42] fulfills the above mentioned requirements. This method was used in paper III for the immobilization of an enzyme-coenzyme complex at a glassy carbon electrode. This approach has the disadvantage of being rather time-consuming due to the excessive number of steps and long reaction times.

Adsorption

Making modified graphite electrodes demands thoroughly understanding of the adsorption mechanism. Studies of selected redox molecules with marked adsorption behaviour can give indications on the molecular structure required. Different orthoquinones 1,2-benzoquinone, 1,2-napthoquinone and 9,10-phenanthroquinone were therefore tested. The first one was very weakly adsorbed

while the third showed an almost total irreversible adsorption. The behaviour of the second was in between these two categories. The number of aromatic rings in the molecule plays a significant role, due to the Π -electron interaction with the graphite surface. Another property which is essential for the stability of the adsorbed layer is the solubility of the molecule in the solution. In aromatic hydrocarbon compounds containing water soluble functionalities like $-\text{COOH}$ and $-\text{SO}_3\text{H}$ groups the rate of desorption increases drastically.

The amount of substance adsorbed can be in certain cases superior to $2 \cdot 10^{-8}$ mole/cm². This is much more than a monolayer, which is the maximum surface coverage obtained for a covalent coupling technique. In relation to the geometrical electrode area a monolayer adsorption of a tricyclic aromatic ring system should correspond to about $3 \cdot 10^{-10}$ mole/cm². The graphite electrodes are polished on a wet fine grade emery paper before use. Micrographs from such electrodes show that the surface has a flake-like and irregular structure. It is obvious that the real surface area then must be much larger than the geometrical area. The increase compared to the geometrical area is very difficult to estimate, but still it probably cannot be only a monolayer coverage. The conclusion must be that at high surface coverage a multilayer adsorption takes place.

The procedure for making CME:s using this technique is very simple. The electrode is dipped into a solution containing the redox compound. The solution can either be water or some

electrode can easily be varied by increasing the time the electrode is in contact with the adsorption solution, the concentration of the redox compound or both. Generally, it is better to adsorb from a weak solution for a long time than the opposite. The surface coverage can then easily be determined electrochemically. The stability of a CME prepared in this way depends of course on the properties of the adsorbed molecule. The main reason for the loss of activity is due to desorption. The rate of this process increases by vigorous stirring of the solution. Increasing of the ionic strength of the electrolyte has a positive effect for the stability. Another reason for the instability can be chemical changes of the molecule at the surface.

3.3 Electrochemistry of CME:s

Cyclic voltammetry (CV) has become a very popular technique for electrochemical studies and it has proved to be very useful in obtaining information about electrode reactions. For a redox modified electrode the reversibility of the system, the amount of adsorption, and the standard redox potential are readily available from a single voltammogram. It is also possible to continuously follow the rate of adsorption or desorption. Theoretical studies of adsorption peaks appearing in CV when both reactant and product are irreversibly attached to the electrode surface have been presented by Laviron [43-46]. The following equations are valid when

- 1) adsorption and electrochemical reactions are assumed to be at equilibrium

2) a Langmuir isotherm is obeyed e.g. all the adsorption sites are equivalent and there are no interactions between the adsorbed molecules.

$$E^{O'} = E^O - \frac{R \cdot T}{n \cdot F} \ln \left(\frac{b_{ox}}{b_{red}} \right) \quad (6)$$

$$E^{O'} = E_{Pox} = E_{Pred} \quad (7)$$

$$i_p = n^2 \cdot F^2 \cdot A \cdot \Gamma \cdot v / R \cdot T \quad (8)$$

$$\delta_{0.5} = (90.6/n) \cdot 10^3 \quad (9)$$

where $E^{O'}$ = standard surface redox potential (V)

b_{ox} , b_{red} = adsorption coefficients

E_{Pox} , E_{Pred} = peak potentials for the oxidation and the reduction waves, respectively (V).

Γ = total superficial concentration (mole $\cdot$ cm $^{-2}$)

A = electron area (cm 2)

v = sweep rate (V $\cdot$ s $^{-1}$)

$\delta_{0.5}$ = width of wave at midheight (V)

i_p = peak current (A)

When conditions 1) and 2) are met, a cyclic scan gives a voltammogram with the oxidation and the reduction peaks symmetrical with respect to the potential axis and with the same peak potential.

Deviation from ideal conditions due to interaction forces between the redox compounds at the surface causes a change in the height and in the width of the peak. When the attraction and the repulsion forces between the adsorbed molecules compensate each other the peak has the shape and properties of the peak when a Langmuir isotherm is obeyed. The peak potential, however, can vary with Γ . The peak becomes narrower and sharper, if the attraction forces predominate and the opposite if the repulsion forces prevail, with increase of Γ .

For an irreversible electrochemical reaction ΔE_p will be > 0 and increases with increasing sweep rate. From plots of E_{pox} and E_{pred} vs sweep rate the electrochemical rate constant k_s and the symmetry coefficient α can be calculated. For an adsorbed multilayer [47] the transmission rate of electrons depends on the layer thickness. An increase of the thickness will slow down the transmission of electrons from the top layer to the electrode due to the increased distance and it will have a moderate effect on the peak height but will result in an intense peak tailing.

For many organic redox compounds the electrochemical reaction also involves protons. The protons must be provided by diffusion or by some buffer component present in solution. At high surface coverage, high pH and low buffer capacity the electrochemical reaction causes a change in pH at the solution electrode interphase. Cyclic voltammograms under these conditions resemble to those of irreversible electrochemical reactions.

For non ideal conditions the redox potential of the system is given by (eq 10)

$$E^{O'} = \frac{E_{Pox} + E_{Pred}}{2} \quad (10)$$

For the compounds studied (papers IV-VI) the surface redox potential is the same as the redox potential in solution. For a $2e^-$, $1H^+$ reaction the $E^{O'}$ will shift by 30 mV/pH unit and for a $2e^-$, $2H^+$ reaction the shift will be 60 mV/pH unit. This method can readily provide data about the number of electrons and protons involved in the electrochemical reaction by varying pH. For substances which are almost insoluble in water the redox potential can easily be determined when the redox compounds are adsorbed at the electrode surface.

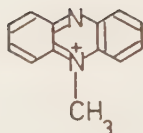
The surface coverage Γ is easily obtained by evaluating the current-time integral under either the oxidation or the reduction wave. Eq (11) can be used, when the number of electrons (n) is known.

$$Q(t) = n \cdot F \cdot A \cdot \Gamma \quad (11)$$

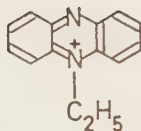
3.4 Choice of redox molecules

In the selection of an electron transfer mediator three main properties are taken into account

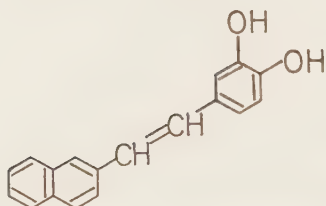
- 1) capability in oxidation of NAD(P)H
- 2) possibility to attach the molecule at an electrode surface
(in this case using the adsorption technique)
- 3) a redox potential considerable more negative than the oxidation potential of NAD(P)H



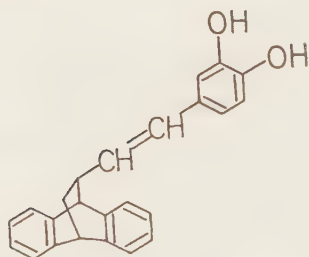
PMS PHENAZINE METHOSULFATE
5-METHYLPHENAZINIUM



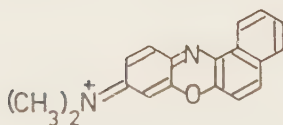
PES PHENAZINE ETHOSULFATE
5-ETHYLPHENAZINIUM



NSCH₂
4-(2-(2-NAPHTHYL)VINYL)CATECHOL



ASCH₂
4-(2-(9,10-ETHANOANTHRACEN-9-
-YL)VINYL)CATECHOL



MELDOLA BLUE, 7-DIMETHYLAMINO-
-1,2-BENZOPHENOXAZINIUM

Fig. 2. Formula for NAD(P)H mediators

The molecules used in this investigation are shown in Fig. 2. PMS^+ , PES^+ and MB^+ (Meldola Blue) are commercially available while NSCH_2 and ASCH_2 were synthesized in our laboratory by Jaegfeldt. The common feature of these redox compounds is that their adsorption to the electrode occurs through the aromatic rings. The electroactive center of the mediators can be divided into three different groups.

- 1) nitrogens in "ortho"-position (PMS^+ , PES^+)
- 2) oxygens in ortho-position (NSCH_2 , ASCH_2)
- 3) nitrogens in para-position (MB^+)

Tse and Kuwana [14] compared ortho-quinones with para-quinones and found that the para-quinones were inactive in the oxidation of NADH. This gives evidence for a structural requirement of the mediator in the oxidation mechanism. No such difference was found between "ortho" and para-diamines (papers IV, VI). Kitani and Miller [48] have also shown that para-phenylenediamine (group 3) can mediate the oxidation of NADH in solution.

Brown et.al. [49] have reported on the adsorption of 9,10-phenanthroquinone, which is closely related to the catechols (group 2). This CME showed no electrocatalytic activity in the oxidation of NADH. For NSCH_2 and ASCH_2 the redox site is supposed to be separated from the electrode surface with an anchor for the attachment. Such a position of the redox site should be more accessible to the reduced coenzyme comparing to a flat adsorption as in the case of 9,10-phenanthroquinone.

3.5 Electrocatalysis

Redox couples capable of oxidizing NAD(P)H can act as an electron transferring mediator (M^+) between the electrode and the coenzyme. This catalysis differs in efficiency and in the shift of potential depending on the mediating system. According to eqs



the catalytic efficiency depends on the rate constant, k_1 (eq 12). The heterogeneous rate constant, k_s , for the adsorbed mediator is assumed to be large compared to k_1 . The net reaction (eq 14) will be the oxidation of NAD(P)H at a decreased overvoltage determined by the redox potential of the mediating system.

In a cyclic voltammogram the current appears as the sum of two contributions

$$i = i_o + i_c \quad (15)$$

where i_o = current obtained without any reactant in solution

i_c = catalytic current

For large k_1 values or at small sweep rates (v) the catalytic current is controlled by mass transfer and a peak-shaped wave proportional to $v^{\frac{1}{2}}$ is obtained [50].

$$i_p = 0.496 \cdot n \cdot F \cdot A \cdot C_{\text{red}} \cdot D_{\text{red}}^{\frac{1}{2}} (n \cdot F / R \cdot T)^{\frac{1}{2}} \cdot v^{\frac{1}{2}} \quad (16)$$

where C_{red} = bulk concentration of the reactant (M).

D_{red} = diffusion coefficient of the reactant ($\text{cm}^2 \cdot \text{s}^{-1}$)

In this case $E_{p/2}$ is identical to $E^{O'}$ for the redox couple attached to the electrode surface. For small k_1 values or at high sweep rates the peak current (i_p) is less than expected from eq (16) and a S-shaped wave is obtained. From measurements of i_p or $E_{p/2} - E^{O'}$ the rate constant can be evaluated [50].

The rotating disk electrode (RDE) can also be used for determining the efficiency and the rate constant, k_1 , for the catalysis of a electrochemical reaction. This method involves convective mass transport of reactants and products. The advantages of using RDE are that a steady state is attained rather quickly and the double-layer charging is negligible. Under totally mass transfer limited conditions the Levich equation is valid

$$i_L = 0.620 \cdot n \cdot F \cdot A \cdot D^{2/3} \cdot \omega^{1/2} \cdot v^{-1/6} \cdot C_{\text{red}} \quad (17)$$

where ω = angular velocity ($\text{rad} \cdot \text{s}^{-1}$)

v = kinematic viscosity ($\text{cm}^2 \cdot \text{s}^{-1}$)

For an irreversible anodic reaction the limiting current is

$$i_a = \frac{n \cdot F \cdot A \cdot k_f \cdot C_{\text{red}}}{1 + k_f \cdot \delta / D_{\text{red}}} \quad (18)$$

where k_f = rate constant ($\text{cm} \cdot \text{s}^{-1}$)

δ = thickness of diffusion layer (cm)

For small k_f -values eq (18) can be written as

$$i_a = n \cdot F \cdot A \cdot k_f \cdot C_{red} \quad (19)$$

For a CME with a fast electrochemical reaction k_f can be exchanged with $k_1 \cdot \Gamma$ and gives

$$i_a = n \cdot F \cdot A \cdot k_1 \cdot \Gamma \cdot C_{red} \quad (20)$$

The catalytic efficiency is obtained as the ratio of the current at the plateau of the catalytic wave and the limiting current (i_l) according to

$$\frac{i_a}{i_l} = \frac{\frac{\rho \cdot k_1 \cdot \Gamma}{D}}{\frac{\rho \cdot k_1 \cdot \Gamma}{D} + 1} \quad (21)$$

$$\text{where } \rho = 1.61 \cdot v^{1/6} \cdot D^{1/3} \cdot \omega^{-1/2}$$

From this eq (21) the rate constant k_1 can easily be evaluated.

Table III shows the modified electrodes used so far for the catalytic oxidation of NAD(P)H. PES^+ has the redox potential closest to the reversible potential of the coenzyme system. The MB^+ modified electrode gives the most negative peak potential due to its large k_1 and negative $E^{O'}$ -value.

The rate constants k_1 are about the same for the PMS^+ and $NSCH_2$ modified electrodes despite their relatively large differences of $E^{O'}$ -values. A straight forward explanation of the interdependence between the rate constant k_1 , the free energy (ΔG) of the chemical reaction and the structure of the mediator cannot be given at this stage.

Table III

Data about CME:s used for the electrocatalysis of NAD(P)H oxidation.

Electrode material	Mediator	$E^{O'}$ vs SCE (pH 7) (V)	rate constant k_1 (M·s ⁻¹)	E_p (V)	ref.
glassy carbon	3,4-dihydroxybenzyl-amine	0.15	-	0.2	(14)
- " -	dopamine	0.17	-	0.2	(51)
carbon paste	1,2-naptho-quinone	-0.15	-	-0.1	(40)
graphite	PMS ⁺	-0.16	10 ³	-0.05	IV
- " -	PES ⁺	-0.21	10 ³	-0.1	IV
- " -	NSCH ₂	0.15	2·10 ³	0.17	V
- " -	ASCH ₂	0.15	-	0.23	V
- " -	MB ⁺	-0.17	6·10 ³	-0.15	VI

4. CONCLUSIONS AND PERSPECTIVES

Chemical modification of electrodes is an increasing research area of recent interest. Its applicability to the electrocatalytical oxidation of reduced coenzymes is well demonstrated. Further knowledge about modification modes, electron transfer mediators and catalytic mechanisms can lead to CME:s capable of oxidizing the coenzyme at the reversible potential. This will be a tremendous progress in the attempt to couple electroanalytical methods with specific enzymatic reactions. Furthermore the oxidation can be used in a regeneration process for biotechnical purposes. It is also possible to construct a bioanode for use in a biochemical fuel cell.

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ELECTROCHEMICAL OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DIRECTLY AND AFTER REDUCTION IN AN ENZYME REACTOR

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SUMMARY

Reduced nicotinamide adenine dinucleotide (NADH) was oxidized coulometrically at a rotating platinum gauze electrode at 0.7 V vs. SCE at pH 9. The NAD^+ formed was reduced enzymatically in a reactor containing immobilized alcohol dehydrogenase. Several recyclings were made with the same lot of NADH. The coulometric results were in reasonable agreement with spectrophotometric measurements. The overall conversion to enzymatically active NAD^+ showed a current efficiency of 99.3%. The electrode pretreatment described is essential for high current efficiency. A continuous method of oxidation of alcohol with electrolytic regeneration of cofactor and removal of aldehyde by dialysis was tested.

Regeneration of cofactors is of some importance both in enzyme technology and for analytical purposes. Two enzymatic reactions, one for the reduction and one for the oxidation of the cofactors, can be coupled to facilitate regeneration of expensive cofactors. For technical purposes, the disadvantage of this method is that the product is mixed with the reagents and products for the regeneration cycle. For analytical purposes, the regenerating system must be chosen so that selective detection is possible, and the equilibrium conditions must be favourable for the desired reaction. An electrolytic regeneration should have advantages in both applications.

Preliminary experiments by Burnett and Underwood [1] showed quantitative recovery of nicotinamide adenine dinucleotide (NAD^+) upon electrolytic oxidation of NADH. However, it was found later [2] that the electrode surface became fouled by accumulation of unknown oxidation products. Aizawa et al. [3] studied the voltammetric properties at platinum electrodes and also performed coulometric oxidations at 0.7 V vs. SCE. The electrolytic cell was also incorporated into a continuous-flow reactor system [4]. A slow adsorption was reported and the current efficiency was 95% [4] for reoxidation. The voltammetric and coulometric oxidation of NADH and other cofactors at glassy carbon electrodes was studied by Braun et al. [5]. An n value of 1.5 was found with controlled potential coulometry

(which corresponds to 75% current efficiency), in contrast to the value of 2.0 found from voltammetric measurements. Blaedel and Jenkins [6, 7] used steady-state voltammetry to study the conditions for direct amperometric determination of NADH in micromolar concentrations. This method was selected as it had been found impossible to oxidize NADH cleanly in aqueous systems by conventional voltammetry. It was concluded [7] that platinum was not quite as suitable as glassy carbon for studies of NADH oxidation; it was also shown that even small amounts of Tris buffer blocked the electron transfer. Thomas and Christian [8] devised an amperometric method for alcohol determination using electrochemical oxidation of NADH. Wallace et al. [9] used NADH oxidation for enzyme determinations in a flow system. Blaedel and Jenkins [10] described a lactate electrode in which the NADH was regenerated electrochemically.

In most of the references cited some difficulties have been encountered in the oxidation, and various methods of electrode pretreatment have been devised. The reasons for the lack of electrode reproducibility are not well understood although adsorption may be of some importance. Why a given pretreatment should change the adsorption behaviour is not easy to understand.

The present investigation is a continuation of the investigation by Aizawa et al. [3] and the condition of the electrolysis is similar. In addition to the spectrophotometric method used by these workers, a coulometric method capable of high precision is included in the present study. The continuous regeneration unit also follows a design described earlier [4] but the conditions used in this work allowed a simultaneous and precise coulometric measurement.

EXPERIMENTAL

Electrochemical oxidation of NADH was done in a three-compartment cell as shown in Fig. 1. Sample was added to the working electrode compartment which contained a rotating platinum-gauze electrode; the gauze was 36 mesh and the electrode diameter was 35 mm, the rotating speed was 550 r.p.m. A mercury contact was provided at the top of the axis. A calomel reference electrode (Radiometer K 401) was placed as close as possible to the rotating electrode. The volume of the chamber was about 20 ml but usually only 15 ml of solution was dispensed into it. Electrolytic connection to the auxiliary electrode was made through two clay-filters with an intermediate chamber in between. The cell was connected to a three-electrode potentiostat with electronic integration and direct read-out of the current-time integral on a digital voltmeter.

The electrolyte was either 0.1 M or 0.05 M sodium pyrophosphate which was adjusted to pH 9.00 with 5 M HCl. Various amounts of NADH (Sigma Chemicals Company, Cat. No. N-8129) were added to the solution in the working electrode compartment. In some experiments ethanol was present and in this case an ethanolic buffer was used in all three compartments.

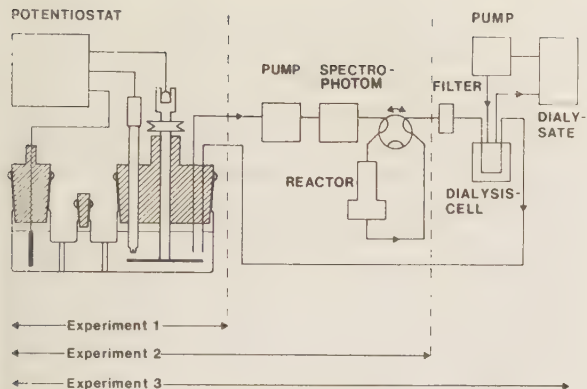


Fig. 1. Diagram of experimental arrangement showing which parts were used during the three reported reactions.

Figure 1 shows the experimental arrangement. The first series of coulometric oxidations was made in the cell, the left part of Fig. 1, with no external devices connected. In the next series of experiments, an enzyme reactor containing immobilized alcohol dehydrogenase was added in an external loop. A peristaltic pump (LKB 12000 Varioperpex) pumped the solution at a flow rate of 5 ml min^{-1} . A flow-through 10-mm spectrophotometric cell was also inserted into the loop. The spectrophotometer was a Zeiss PMQ II. The enzyme reactor could be bypassed by switching a sample loop (Altex Rotary Valves, Series 202) which had a volume of 2.7 ml. The third arrangement included a hollow-fiber dialysis cell (Bio-Rad Laboratories, model Bio-Fiber 20 Beaker) with a cut-off at 200D. The dialysate consisted of 1100 ml of exactly the same buffer solution as that filled into the loop and electrolysis cell except that no NADH was present in the dialysing solution. The solution was pumped through the hollow-fiber unit at 10 ml min^{-1} . The volume of the external cell loop was increased by about 4 ml when the dialysis cell was inserted. The loop also contained a Millipore filter unit (Swinnex-13 model) with a $5\text{-}\mu\text{m}$ cellulose acetate filter to prevent clogging of the hollow fibers.

The alcohol dehydrogenase (Sigma Chemical Company, Cat. No. A-7011) was immobilized on porous glass (CPG-10/700 Å, 80–120 mesh, pore diameter 654 Å; Serva Feinbiochemica) with glutaraldehyde as described earlier [11]. The immobilized enzyme was filled into a reactor (i.d. 5 mm, length 55 mm) and held with $5\text{-}\mu\text{m}$ Millipore filter discs at the lower end. The porous glass was not an ideal support and the reactor became deactivated several times. Alumina would probably have been a better support [4].

RESULTS

Single-pass electrolysis

NADH was dissolved in 0.1 M sodium pyrophosphate to give a stock solution of about 0.15 mM. A more exact determination of the concentration was made spectrophotometrically, based on a molar absorptivity of $6220 \text{ l mol}^{-1} \text{ cm}^{-1}$ [12]. An aliquot of the stock solution was added to the coulometric cell and oxidized at 0.70 V vs. SCE. The results are shown in Table 1. Some experiments were also made in which a smaller aliquot was taken; the volume was made up with buffer. It can be seen that the coulometric and the spectrophotometric determinations give the same result within 1.3% or better. This shows that a coulometric oxidation of NADH can be made quantitatively, in contrast to some earlier reports [1, 4, 5].

In order to obtain these results, it was necessary to pretreat the working electrode by first washing it in warm, concentrated nitric acid for about 10 min, then rinsing thoroughly in water, and preelectrolysing in 0.1 M pyrophosphate buffer at 0.7 V until the residual current decreased to a value of 10–20 μA . The pretreatment changed the oxidation behaviour drastically. The electrolysis current in both cases followed the equation

$$i_t = i_0 e^{-\beta \cdot t} \quad (1)$$

in which i_t is the current at the time t , e the natural logarithm base, and β and i_0 are constants. After preelectrolysis, values of β were around $8 \times 10^{-3} \text{ s}^{-1}$, but without the pretreatment the value of β varied, often being much lower, down to $1 \times 10^{-3} \text{ s}^{-1}$. The determinations made after pretreatment indicated 100% current efficiency, i.e. the amount of NADH calculated from the current integral was equal to the amount determined spectrophotometrically. Without pretreatment the current efficiency varied; values of 65%, 116%, 110% as well as some around 100% were obtained.

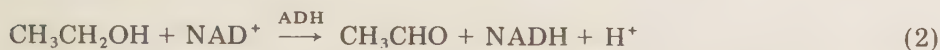
TABLE 1

Comparison of spectrophotometric (340 nm, $\epsilon = 6220 \text{ l mol}^{-1} \text{ cm}^{-1}$) and coulometric (Pt, pH 9.0 pyrophosphate, +0.70 V vs. SCE) determination of NADH

NADH (μmol)		Number of runs	Difference $\Delta\%$
Spectrophotometric	Coulometric		
0.79	0.78	1	1.3
0.83	0.82	1	1.2
1.57	1.58	4	-0.6
1.61	1.61	3	0
1.63	1.63	2	0
1.65	1.65	3	0
2.47	2.45	2	0.8

Electrolysis and enzymatic regeneration

For a successful electrolytic oxidation of a cofactor the oxidation product must necessarily be enzymatically active. Alcohol dehydrogenase is dependent on NAD^+ for the reaction



By adding immobilized ADH in a side loop it should be possible to regenerate NADH quantitatively from NAD^+ . Oxidation products other than NAD^+ should not be reduced and it should therefore be possible to achieve a quantitative measure of the inactive side products. In order to increase the accuracy of the measurement, the set-up was arranged so that the same lot of NADH could be electrolytically oxidized, enzymatically reduced, electrolytically oxidized etc. Figure 2 shows the results obtained during such a recycling when the original concentration of ethanol was 0.53 M in 0.1 M pyrophosphate, pH 9.0. The circles give the amount of NADH oxidized as a percentage of the amount ($107 \mu\text{mol}$) originally present.

There is a steady decrease of the amount of NADH which is regenerated. It is known that NAD^+ is unstable in alkaline solution whereas NADH is more stable. The rate of this non-electrolytic decomposition was measured separately by storing a solution of buffer- NAD^+ at pH 9.0 (see below). A complete regeneration of NADH will also be prevented if the acetaldehyde concentration increases. The equilibrium constant of eqn. (2) is known [4]; it was taken to be 2.31×10^{-2} when the H^+ concentration is included. The concentration of NADH after the i th oxidation will then be

$$[\text{NADH}]_i = -X + (X^2 + Y)^{\frac{1}{2}} \quad (3)$$

$$X = ([\text{EtOH}]_i + [\text{NAD}^+] + [\text{CH}_3\text{CHO}]_i) / 2.31 \times 10^{-2} / 84.6$$

$$Y = [\text{EtOH}]_i [\text{NAD}^+]_i / 42.3$$

The acetaldehyde present as an impurity in the alcohol was estimated to be $80 \mu\text{M}$ at the start; the amount was determined by gas chromatography (Varian Aerograph 2700, FID, 4-m column, i.d. 2 mm, 15% PEG on Chromosorb, 65°C). Equation (3) was evaluated by computer with the $80 \mu\text{M}$ start concentration of aldehyde and the result is given in Fig. 2, curve A. Obviously equilibrium considerations alone cannot explain the experimental results.

The non-electrolytic decomposition of NAD^+ , measured spectrophotometrically, as above, was found to be $6.1 \times 10^{-5} \text{ min}^{-1}$. Each cycle took 52 min and therefore the decomposition can be approximated by the equation

$$[\text{NAD}^+]_i = [\text{NAD}^+]_0 (1 - ki) \quad (4)$$

where $k = 1.6 \times 10^{-3}$. Curve B, Fig. 2, shows the result when both the non-electrolytic decomposition and the equilibrium were taken into account.

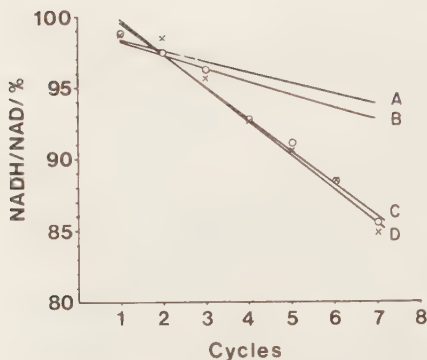


Fig. 2. Recovery of NADH vs. the number of oxidation cycles. Experiment 2. (A) Calculated from equilibrium data. (B) As for A but including pH-dependent non-electrolytic decomposition of NAD^+ . (C) Measured coulometrically ($\circ$). (D) Measured spectrophotometrically ($\times$).

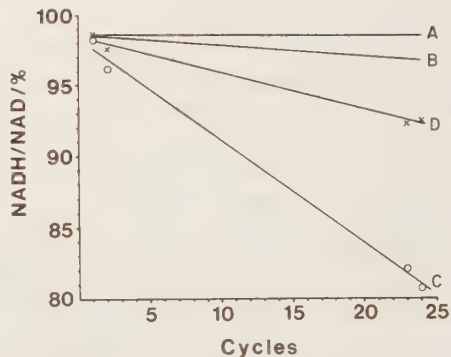


Fig. 3. Recovery of NADH vs. number of reoxidations when dialysis was used. Experiment 3. Legends to curves A–D as in Fig. 2.

Curves C and D represent the least-squares lines through the experimental points. The difference between curves B and C and line D is thus a measure of decomposition at the electrode which is estimated to be 1% per cycle.

Recycling with dialysis

To reduce the concentration of acetaldehyde to such a low value that eqn. (2) can be considered to proceed almost completely to the right, a dialysis cell was included in the loop (Fig. 1). The products which could pass the hollow fibers were diluted to a total volume of 1100 ml as compared to the 17 ml in the electrolysis cell, the loop and the hollow fiber unit. The acetaldehyde was thus diluted 65 times compared to the experiment reported above. Some low-molecular-weight products, probably decomposition products from the cofactor, also passed into the dialysate. It is not known if those affect the oxidation. The external solution was periodically checked for the presence of NAD^+ and NADH with a spectrophotometer (McPherson model EU-707-11). No NAD^+ or NADH could be found with the method which had a detection limit of $0.08 \mu\text{mol}$ corresponding to a 6% loss during a run of 9 h 22 min. The sensitivity of the test was thus too low to detect minor losses.

Figure 3 shows the results obtained when $1.34 \mu\text{mol}$ of NADH was dissolved in 0.05 M pyrophosphate buffer, pH 9.0, containing 0.53 M ethanol and $80 \mu\text{M}$ acetaldehyde as an impurity. The total volume was 17 ml. First a complete oxidation was performed with the enzyme reactor bypassed (cycle 1). The enzyme reactor was then used to reduce the NAD^+ completely with the potentiostat switched to stand-by, and the oxidation cycle was then repeated once more (cycle 2). Then the valve was switched

to allow continuous operation. The current integral was taken as a measure of the number of recyclings. After about twenty passes the system was switched back to discontinuous operation to obtain the amount of active cofactor.

Curve C in Fig. 3 is drawn through the points obtained by coulometry and curve D through those obtained spectrophotometrically. Curve A gives the amount expected when the equilibrium constant is taken into account as described above. The line is practically parallel (0.2%/24 cycles) to the abscissa but less than 100% because of the acetaldehyde impurity. Curve B includes also the non-electrolytic decomposition of NAD^+ at pH 9, obtained as above but with a mean cycle time of 23 min during the time of continuous operation.

The two methods give different values. The reason for this lies probably in the different modes of operation. The spectrophotometric method measures a concentration whereas the coulometric method primarily measures an amount (of electricity). Any decrease in volume, e.g. by reverse osmosis through the hollow fibers, could give such a discrepancy with the coulometric method producing the lower value. It was difficult to measure the volume precisely.

The overall result is that NADH can be oxidized electrolytically to enzymatically active NAD^+ with an overall efficiency of 99.3%. This value is obtained when the results from the coulometric method (curve C, Fig. 3) are used.

DISCUSSION

The present results indicate that a quantitative reoxidation of NADH to NAD^+ is possible with a very high current efficiency in a two-electron process; and the product is enzymatically active. These results contrast with some earlier reports in which substantially lower current efficiency was found. Electrode fouling has also been reported [2, 9] as well as adsorption and different behaviour on successive sweeps in voltammetry [3]. The oxidation peak in voltammetry appears at +0.7 V but the half-wave potential for reduction occurs in two steps, 0.9 and 1.7 V [1] at the DME. This indicates a complex electrochemical behaviour.

The pretreatment of the platinum electrode was absolutely essential in order to obtain reproducible and accurate results. The condition of the electrode surface must therefore be an essential factor for the quantitative oxidation of NADH. Probably the condition of the surface affects the adsorption behaviour but the exact mechanism has not been studied further. Another factor which might influence the result is the mass transfer at the working electrode. In the cell used, the platinum gauze was rotated at high speed. A violent turbulence is created around each wire in the mesh and the Nernst diffusion layer is therefore very thin. Any byproducts from the oxidation will thus be transferred into the bulk of the solution very rapidly.

The concentration of active products at the electrode surface will therefore be much lower than if the electrolysis were performed at a stationary or plane electrode. This may be a factor in explaining why no electrode fouling was observed even after very long repeated oxidation.

The accuracy of the coulometric measurements is rather astonishing when the procedure is considered. Diffusion of reactant will normally take place through the filters and during a long run an appreciable loss can occur [13]. The cofactor could diffuse into the intermediate chamber and the counter electrode compartment. The intermediate chamber was occasionally analyzed spectrophotometrically and in all cases the amounts of NAD^+ and NADH were negligible. Both NAD^+ and NADH have a negative overall charge and therefore the migration counteracts diffusion. This effect should be small, however, as the electrolyte concentration is fairly high. Possibly the clay filters are charged so that exclusion takes place and the charge is transported by positive ions.

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Regular papers**235 - Spectroscopic and Theoretical Studies
on the Intramolecular Electron Transfer in NAD**

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Manuscript received February 13th 1978

Summary

The feasibility of modifying a natural coenzyme (oxidised nicotinamide adenine dinucleotide, NAD^+) to facilitate electron transfer to and from an electrode has been discussed. The present study suggests that the adenine and nicotinamide moieties in NAD^+ interact too weakly for an effective electron transfer from nicotinamide to adenine to take place. The geometry of the molecule, calculated from published results on preferred conformations obtained from theoretical, NMR and X-ray crystallographic studies, is generally unfavourable for a direct electron transfer to occur. It is concluded that substitution on the adenine ring will not enhance the electron transfer properties of the molecule to any appreciable extent.

Introduction

A bioelectrode, particularly a bioanode, has been considered seriously for use in a biochemical fuel cell. In a biochemical fuel cell, the catalyst used for oxidation of the fuel is an active enzyme that has obvious advantages over conventional noble metal catalysts.¹ The most difficult part in the design of a suitable bioanode is the slow transfer of electron from the fuel to the electrode.² The oxidation process needs a coenzyme, that undergoes concurrent reduction. Although the coenzymes possess some electroactivity they often show irreversible redox behavior at the electrode. In a previous report, a few modified coenzymes were considered for a practical bioanode.³

Nicotinamide adenine dinucleotide is an unusual dinucleotide in the sense that it contains a 5'-5' linked pyrophosphate unit (Fig. 1). It has been suggested that the adenine moiety is mainly responsible

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for the stereospecificity of the enzymatic reduction and binding with the enzyme.⁴ The nicotinamide unit is responsible for the redox properties of the coenzyme⁵ and the electrochemical reduction of NAD^+ is neither reversible nor selective.^{6,7}

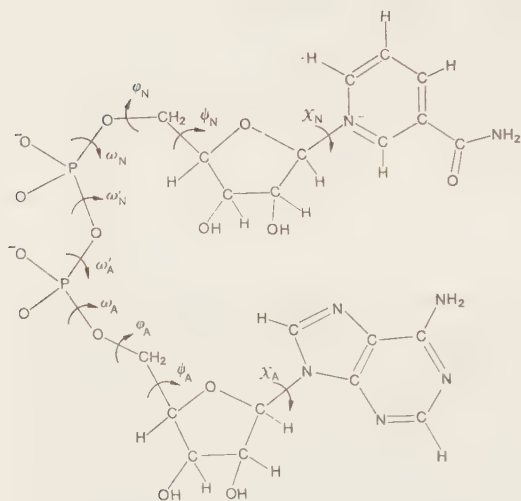


Fig. 1.

The schematic diagram for NAD^+ with the torsional angles indicated therein.

SHIFRIN has found evidence from spectroscopic studies that the coenzyme (NAD^+) can interact with amino-acid side chains (tryptophan) by a charge transfer interaction and this has been suggested as responsible for binding to the enzyme.⁸⁻¹⁰ The basic theory for charge transfer interaction in molecular systems was developed by MULLIKEN around 1950.^{11,12} We have attempted here to study the intramolecular charge transfer interaction, if any, between the nicotinamide and the adenine moieties in NAD^+ .

Theoretical

PULLMAN and PULLMAN¹³ have shown that electronic parameters calculated for NAD^+ can be correlated with their macroscopic chemical properties. More recently the preferred conformations for NADH has been obtained by a more rigorous quantum-chemical formalism.¹⁴ Using their predicted set of conformational angles, we have obtained the molecular geometries for NAD^+ and calculated the distance and angle between the two planes represented by the two bases. The sugar conformation was assumed to be $\text{C2}'\text{-endo}$ and the geometry for $\text{C2}'\text{-endo}$ sugar was obtained from the works of ARNOTT and HUCKINS.¹⁵ The torsional angles are the same as in the original reference.¹⁴ These results are listed in Table I.

Table 1. Interplanar distances and angles for various conformations of NAD⁺. (The angles refer to Fig. 1).

Conf. no.	Ψ_N	ω_N	ω'_N	ω'_A	ω_A	Ψ_A	Distance*	Angle**
1	330	300	300	330	300	330	14.86	68.02
2	60	60	30	60	60	60	12.80	79.36
3	60	60	30	90	300	60	12.27	62.25
4	330	330	330	270	60	60	4.80	9.46
5	60	60	270	330	300	330	11.48	15.88
6	60	60	30	210	60	60	13.47	79.6
7	60	60	30	60	60	330	12.21	85.23
8	330	300	330	270	300	60	13.21	52.19
9	330	60	270	330	300	330	11.55	73.09
10	60	300	270	330	300	330	12.26	80.52
11	330	300	330	270	60	330	13.16	51.70
12	272	109	37	204	104	285	9.98	23.06
13	318	85	49	204	107	285	10.49	50.35
14	60	300	300	120	60	60	9.62	71.57
15	60	300	300	270	60	330	10.13	59.56

* Distance refers to the centre to centre distance between the two bases in Angströms.

** All angles are reported in degrees.

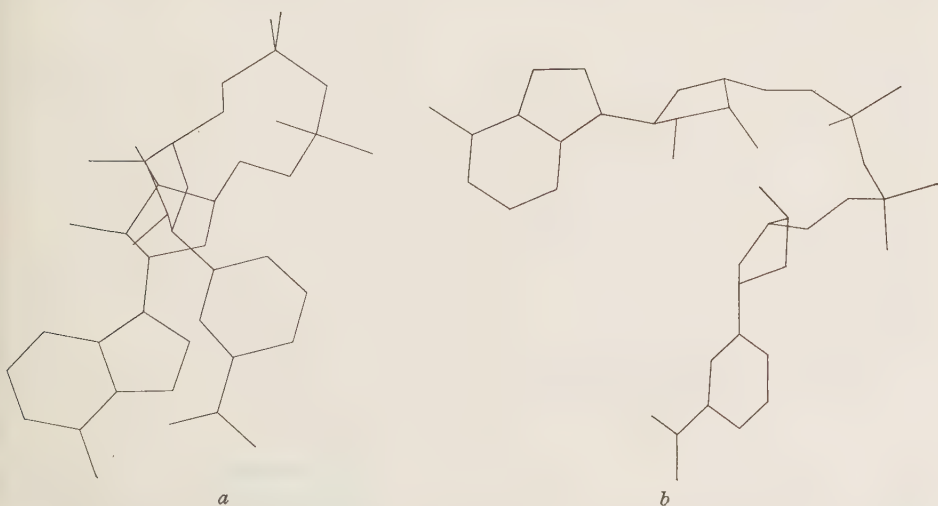


Fig. 2.

a) A scale diagram for NAD⁺ for the conformation no. 4 with the torsional angles given in Table I.

b) A scale diagram for NAD⁺ as found from X-ray studies on NAD⁺-LDH binary complex. The torsional angles correspond to conformation no. 12 in Table I.

Although several folded and several extended conformations were predicted, all, except the fourth conformation (Table 1), show quite large interplanar distance and angle. The fourth conformation is an extremely folded one and the interbase distance is about 4.8 Å and the interbase angle is about 9.6°. However, the PCILO energy for this conformation is about 3.2 kcal/mole and consequently, the probability for this conformation may be quite low.¹⁴ However, the energy reported may not be very reliable and may further be modified by interaction with the solvent. Fig. 2 shows the fourth and twelfth conformations. These reasons prompted us to undertake an experimental investigation to study intramolecular charge transfer in NAD^+ .

Spectroscopic studies

The near UV absorption spectra in a 0.05 *M* phosphate buffer (pH 5.0) for NAD^+ , NMN^+ and AMP have been obtained from a recording spectrophotometer (GCA/McPHERSON Instrument, model EU-700) in the wavelength region 250–320 nm for concentrations of 1 mM, 0.5 mM and 0.1 mM. The β NAD^+ (Cat. no. N-7004), β NMN^+ (Cat. no. N-3501) and AMP (Cat. no. A-1752) were purchased from SIGMA CHEMICAL Co, and were used without further purifications. The concentrations were determined gravimetrically and correction factors for purity ($\text{NMN} = 98\%$, AMP and NAD, 99%) were applied. The agreement of the observed molar absorptivity with the literature values is excellent for NAD and AMP.

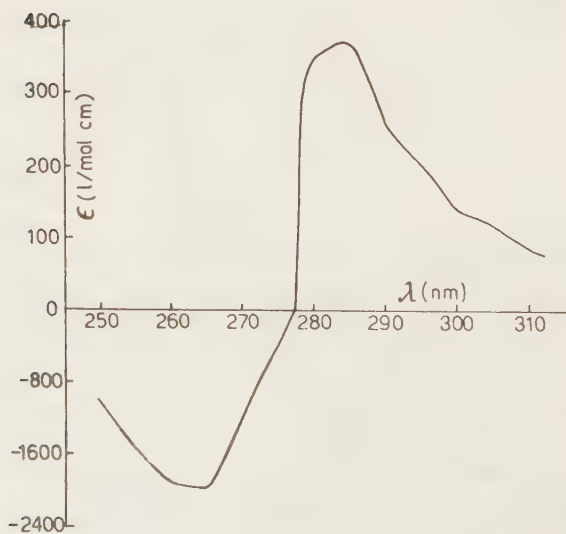


Fig. 3.

The difference spectrum for NAD^+ ($\epsilon(\text{NAD}^+) - \epsilon(\text{NMN}^+) - \epsilon(\text{AMP})$). Note the difference in scale on the positive and negative sides of molar absorptivity.

The difference spectrum was computed manually ($\epsilon_{\text{NAD}^+} - \epsilon_{\text{NMN}^+} - \epsilon_{\text{AMP}}$) at intervals of 5 nm (Fig. 3). This spectrum shows negative absorbance up to 275 nm (*i.e.*, AMP and NMN together absorb stronger than NAD^+ in this region) and a weak positive absorbance from 280–320 nm (*i.e.*, NAD^+ absorbs stronger than AMP and NMN⁺ together). The negative absorption may be ascribed to a hypochromic effect. However, the positive absorption is too weak to be definitely ascribed to a charge transfer interaction.

Conclusions

WEBER has reported from fluorometric studies in NADH in aqueous solution an interaction between the dihydronicotinamide and the adenine moieties,¹⁶ and this has subsequently been confirmed by SHIFRIN and KAPLAN.¹⁷ However, in fluorescence, the excitation energy can be transferred over rather large distances ($\sim 50 \text{ \AA}$). Nevertheless, this interaction is clearly not of charge transfer type and possibly cannot be exploited to produce reversible electron transfer between these two functional subunits.

Interestingly, the only conformation that can give rise to a charge transfer interaction (conformation no. 4 in Table 1 and *a* in Fig. 2) is either totally absent or present in too low a concentration to produce a detectable effect. The energy of this conformation, as suggested by PERAHIA *et al.*, suggests a population of $\sim 1\%$. Alternatively, although the geometry is favourable, the charge distribution may not be favourable.

Some preliminary results on the conformation of NAD^+ bound to lactate dehydrogenase are also available from X-ray crystallographic studies.¹⁸ The geometrical parameters for the binary complex of NAD^+ with LDH and for the ternary complex with LDH and pyruvate are also available. These are reported in conformation nos. 12 and 13 (Table 1 and Fig. 2*b*) and it can be seen that the orientation of the bases is unfavourable for a charge transfer interaction to take place.

SARMA and MYNOTT have been able to predict the possible conformations for NAD^+ using FTNMR in aqueous solutions.¹⁹ Two of the conformations predicted by them are reported in Table 1 (conformation nos. 14 and 15). It can be seen that the geometry of these conformations too are unfavourable for a charge transfer interaction. However short-lived more favourable conformations may exist. The attachment of the cofactor to a solid support may also result in other probable conformations than those given in Table 1.

The broad conclusion that can be drawn from this investigation is that the modification of the adenine moiety in NAD^+ is likely to be of little help in designing a reversible bioanode using covalently immobilized NAD^+ systems. However, modification of the nicotinamide moiety is more promising at this moment and attempts are underway in this direction.

Acknowledgements

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ELECTROCHEMICAL REGENERATION OF NAD COVALENTLY BOUND
TO LIVER ALCOHOL DEHYDROGENASE

KEY WORDS: NAD Regeneration, Modified Electrode,
LADH-NAD Complex

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ABSTRACT

An enzyme-coenzyme complex was immobilized on the surface of a glassy carbon electrode and investigated with cyclic voltammetry in ethanol-containing buffers. The complex consists of an Liver Alcohol Dehydrogenase molecule to which an NAD-analogue is covalently attached via its straight six-carbon spacer. One cycle was observed but repeated recycling could not be carried out, presumably due to catalytic decomposition of the coenzyme⁻ at the electrode surface.

INTRODUCTION

The successful covalent binding of NAD^+ to liver alcohol dehydrogenase (LADH) via a spacer¹ opens up the possibilities for new regeneration methods. This work reports about attempts to regenerate such an enzyme-bound coenzyme electrochemically, especially after its immobilization to a glassy carbon electrode surface.

Related reports have been made by Blaedel et al² who polymerized NAD^+ and Lactate Dehydrogenase around an electrode surface. The electrochemical oxidation of soluble NADH at graphite electrodes has been studied by Moiroux et al³ and at platinum electrodes by Jaegfeldt⁴.

EXPERIMENTAL

An enzyme-coenzyme complex was prepared according to Månsson et al¹ by coupling the NAD-analogue $\text{NH}_2 - (\text{CH}_2)_6 - \text{NHCOCH}_2 - \text{NAD}^+$ to LADH.⁵

Plates of glassy carbon (Le Carbon-Lorraine, Paris) were polished with carborundum papers and finally lapped with ashfree filter paper. The electrode material was then oxidized in an oxygen plasma, reduced with LiAlH_4 and activated with cyanuric chloride as described by Kuwana et al⁶. The enzyme-coenzyme complex, 4

mg/ml in 50 mM phosphate buffer pH 8.5 was allowed to react with the activated glassy carbon at 4°C during at least 12 h. A similar procedure was also used to immobilize LADH to activated glassy carbon. The glassy carbon was mounted in a holder with an O-ring seal so that a small cup for the solution was obtained with the carbon as a bottom. The available electrode surface was 0.25 cm²; the auxiliary (a Pt wire) and reference (SCE) electrodes contacted the solution from above. Measurements were made using triangular sweep cyclic voltammetry.

RESULTS AND DISCUSSION

The reaction sequence to be tested starts with an enzymatic reduction of the immobilized enzyme-coenzyme complex (Figure 1). An electrochemical oxidation is then made and the amount reacted on the electrode surface is estimated from the difference between the obtained voltammogram and a voltammogram of a reduced glassy carbon electrode. In order to be able to draw conclusions about reactions of the immobilized complex, it was necessary to start with the components in solution and of the immobilized LADH itself.

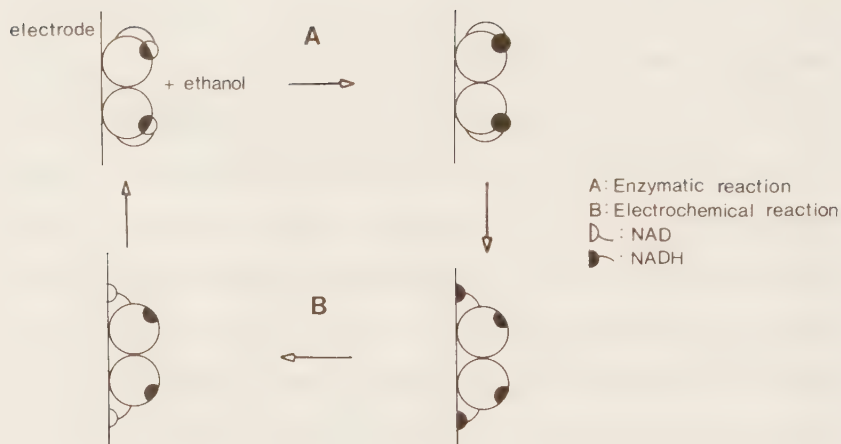


FIG. 1. Schematic drawing of enzyme-coenzyme complex immobilized on the electrode surface. This is an idealized picture however. In reality only one out of four NAD^+ is in a position enabling proper interaction with an active site of the enzyme.

Electrochemical oxidation of NADH and the NADH-analogue in solution

Fig. 2 a shows a cyclic voltammogram obtained in 0.1 M pyrophosphate buffer, pH 9.0, containing 1.0 mM NADH. The electrode was plasma etched and reduced but not activated. There is an oxidation peak at 500 mV vs SCE in close agreement with the results reported earlier³. Fig. 2 b shows a voltammogram obtained with the NADH-analogue obtained under similar conditions except that its concentration was 0.6 mM. The oxidation wave of the analogue is almost identical to that of NADH it-

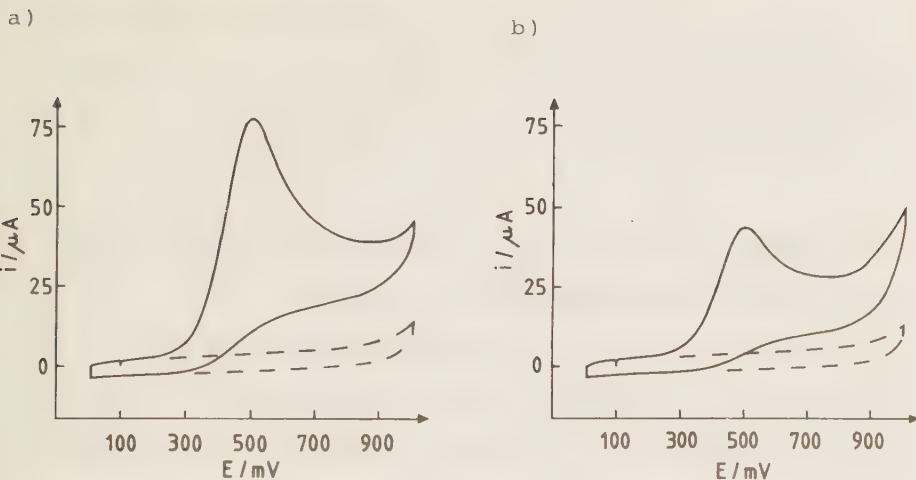


FIG. 2. Cyclic voltammograms of a) 1.0 mM soluble NAD and b) 0.6 mM soluble NADH-analogue, both at pH 9.0. Background currents obtained in the buffer only are shown with broken lines.

self, which is to be expected as substitutions in the adenine part of the molecule should have negligible effect on its electrochemical behavior.

On successive sweep the peak potential waves move slowly in the positive direction. This parallels the earlier findings⁴ on oxidation of NADH on platinum electrodes; the reaction rate decreases and the peak moves due to adsorption of NAD^+ to the electrode surface. If similar experiments were made in the presence of 1.3 mg LADH/ml solution there was a larger displacement towards the anodic side, otherwise the cur-

ves were equal. This indicates that LADH is slowly adsorbed to the electrode so that the number of sites available for oxidation of NADH becomes smaller.

Electrochemical oxidation of NADH formed by LADH immobilized on the electrode surface

Fig. 3 shows a voltammogram obtained in an 0.1 M pyrophosphate solution, pH 9.0, containing 0.2 M ethanol and 1 mM soluble NAD^+ . LADH had been immobilized to the glassy carbon surface using cyanuric chloride. A pronounced peak can be seen in the range 600 -

Fig. 3

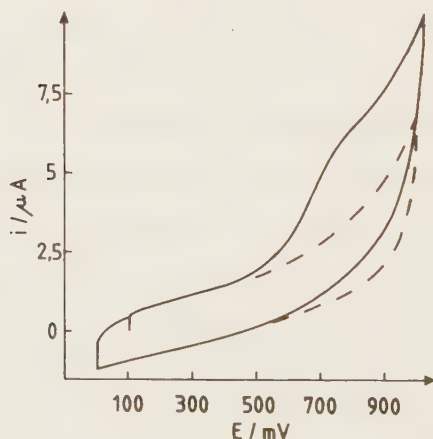


FIG. 3. Cyclic voltammogram using an electrode covered with immobilized LADH 30 minutes after immersion in a solution containing soluble NAD^+ and ethanol.

800 mV vs SCE indicating that NADH has been produced by LADH on the electrode surface. The scan shown in Fig.3 was obtained after 30 minutes in the solution. Peaks were also observed on two scans taken immediately after that shown in the figure but there were no peaks in a fourth scan taken one hour later. The conclusion is that an active enzyme can be coupled to the electrode surface with retained activity. The stability of the enzyme is low however, and the activity is essentially lost at the fourth sweep.

Electrochemical oxidation of soluble enzyme-coenzyme complex

A solution containing 36 μM enzyme-coenzyme complex in an 0.1 M pyrophosphate buffer, pH 9.0, and 0.2 M ethanol was investigated electrochemically. Fig. 4 shows that the enzymatically reduced coenzyme can be oxidized electrochemically. The dashed curve shows the background and if it is subtracted from the main curve a peak current of $2.2 \mu\text{A}/0.25 \text{ cm}^2$ at 600 mV vs SCE was obtained.

The enzyme contains two subunits and only one out of four of the immobilized coenzymes will be in the correct position²; the effective coenzyme concentration will therefore be 18 μM . It is possible to make an es-

Fig. 4.

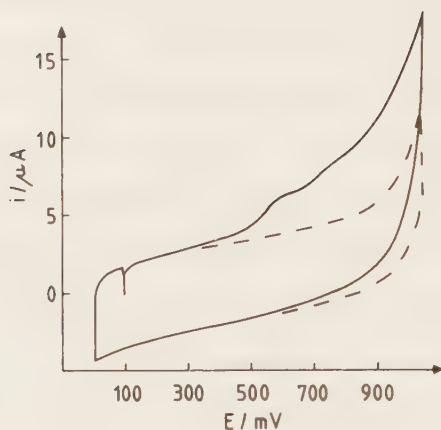


FIG. 4. Cyclic voltammogram using a reduced glassy carbon electrode 5 minutes after immersion in a solution containing the soluble LADH-NADH-complex and ethanol.

timination of the peak current to be expected if the diffusion coefficient and the concentration of coenzyme is known.

The diffusion coefficients were estimated by the Stokes-Einstein relation and the result was $7.6 \cdot 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$ for the enzyme bound NADH complex and $3.7 \cdot 10^{-6} \text{ cm}^2$ for free NADH. The peak current of an NADH-solution can be determined from Fig. 2 a and after adjustments for the different concentrations it can be concluded that a peak current of $0.6 \mu\text{A}$ should be expected in the experiment shown in Fig. 4. The actual value $2.2 \mu\text{A}$ is thus more than three times higher than the expected

value. The explanation is probably that the enzyme-coenzyme complex is attracted to or adsorbed on the electrode surface giving a higher local concentration than that of the bulk of the solution.

Electrochemically the reactions of the enzyme bound NADH seem to parallell those of the soluble NADH fairly well.

Oxidation of immobilized enzyme-coenzyme complex

An electrode covered with the immobilized enzyme-coenzyme complex was washed well with buffer and investigated in 0.1 M pyrophosphate, pH 9.0, also containing 0.2 M ethanol. Fig. 5, curve A shows the first scan taken about 10 minutes after the electrode was immersed in the solution. Compared to a background scan there is a significant increase in current in the range 500-800 mV vs SCE. The effect was reproduced well on a number of electrodes.

The background scan was made on an electrode treated in the same way except that the final step, immersion in an enzyme-coenzyme complex solution, was omitted. In separate experiments it was found that the presence of only LADH gave a scan identical to this background.

Curve B in Fig. 5 shows a second scan obtained after 15 minutes more in the solution. The potential during the waiting was kept at + 100 mV. At this current sensitivity each scan will lower the background somewhat more. If this effect is taken into account curve B equals a scan of a pure background. The broad peak observed on the first scan is absent in the second and in all following scans. This behavior was consistently the same for different batches and for different waiting times.

Soluble NAD^+ (1 mM) was added next in order to see if the enzyme was still present and active and then the

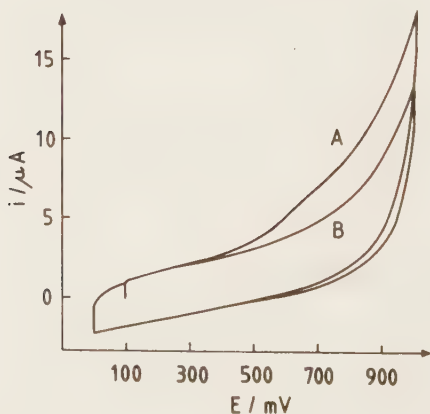


FIG. 5. Cyclic voltammogram using an electrode covered with the immobilized ADH-NADH-complex and immersed in a solution containing ethanol. A 10 minutes after immersion and B 15 minutes later.

broad peak at 600-900 mV could be seen again. The peak could be observed on successive scans in the same way as shown in Fig. 3.

The current-time integral of the shoulder in Fig. 5 was estimated to 3 microcoulombs. If the size of the enzyme is $45 \times 60 \times 110 \text{ \AA}^3$ and the $45 \times 60 \text{ \AA}$ surface is supposed to cover the electrode the expected amount of electricity should be 0.07 microcoulombs. The surface of the glassy carbon electrode was studied by electron microscopy and it was found that the plasma etching produced a substantial increase in the area. The visual impression is that the observed surface roughness at a magnification of 10 000 times only can account for a factor in increased area of 5 or possibly 10. There might be additional roughness on a still smaller scale so that the amount of immobilized enzyme becomes 40 times larger than that calculated from a single-layer coverage of the geometrical surface.

Mechanisms of inactivation of the enzyme-coenzyme complex

Adsorption of oxidized soluble NAD^+ at electrodes has been well documented in the literature. Since the concentration of NAD^+ -groups is small with the immobilized complex, all of the coenzyme might bind to the ac-

tive surface sites of the electrode. The attachment may be so strong that the cycle stops.

The other explanation considered is that the co-enzyme decomposes at the electrode surface. It has been shown earlier⁸ that NADH can be decomposed or oxidized catalytically at graphite surfaces. Similar measurements were made on the glassy carbon electrodes used in this study in order to find out the rate of the decomposition. Pieces of the electrode material was immersed in solutions containing soluble NADH. The solutions were analyzed with HPLC after various times; both NAD^+ and fragments from the decomposition being measured. It was found that the polished glassy carbon itself had only a small effect. The amount of NAD^+ and decomposition products was almost equal to that of the blank, consisting of buffer without any electrode present. The plasma oxidized electrode material produced mostly NAD^+ whereas the reduced electrode material produced mostly decomposition products. The latter corresponds closely to the surface behind the immobilized LADH-NADH-complex. The decomposition rate was of the order of five monolayers NADH per hour.

The conclusion from the mentioned experiment is that the catalytic decomposition alone is sufficient to explain the short life-time of the electrode.

In conclusion, we have demonstrated that the reduced NAD^+ -analogue alone or when coupled to Liver Alcohol Dehydrogenase can be electrochemically oxidized. Also when the enzyme-coenzyme complex was immobilized directly on the electrode the reduced coenzyme could be oxidized, but only once. Proper modifications of the electrode surface might, however, allow further cycles.

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CATALYTIC OXIDATION OF NADH BY SURFACE-MODIFIED GRAPHITE ELECTRODES

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ABSTRACT

A voltage-facilitated immobilization procedure for phenazine methosulphate (PMS) and phenazine ethosulphate (PES) on graphite electrodes is described. The graphite is immersed in a solution of PMS or PES and a voltage of 1.1 V vs. SCE is applied for 15 min. The surface coverage was 5×10^{-9} mol cm⁻². At pH 7.0 the $E_{1/2}$ for immobilized PMS (–160 mV) and PES (–210 mV) were the same as for the soluble compounds.

The modified electrodes catalysed electron transfer from dihydronicotinamide adenine dinucleotide (NADH). Linear sweep voltammetry produced anodic peaks of the NADH oxidation at –50 mV (PMS electrode) and –100 mV (PES electrode). The electrochemical step was faster than the preceding chemical step involving a reaction between NADH and the immobilized PMS or PES.

INTRODUCTION

The electrochemical oxidation of NADH has been studied in several papers [1–4]. It proceeds irreversibly at all known electrode materials. In aqueous solutions at pH 7 the overvoltage is about 1 V at both platinum [4] and carbon [5] electrodes. The reversible potential for the oxidation of the nicotinamide part of the molecule should change with 30 mV/pH. However, the actual oxidation potential varies little with pH [6]. The main product of the oxidation is enzymatically active NAD⁺ [7].

Only a few redox couples can exchange electrons with NADH in a homogeneous solution. Flavines are known to be very slow mediators [8]. With incorporation into some flavine-containing enzymes, e.g. xanthine oxidase [9], the rate of electron transfer increases substantially. The phenazines constitute another group of compounds which can act as fairly good mediators. Examples from this group are the phenazine methosulphate (PMS) [10,11] and the phenazine ethosulphate (PES) [12]. Also, some phenothiazines are active mediators e.g. methylene blue [13], thionine and toluidine blue. Tse and Kuwana [14] found that several 1,2-quinones were quite efficient electron-transfer mediators. 2,6-Dichlorophenolindophenol [8] and some NAD analogues [8] should be mentioned in this connection.

Attempts have been made to use some of the molecular structures mentioned above on surface-modified electrodes in order to speed up the heterogeneous

electron transfer between an electrode and NADH in solution. Tse and Kuwana [14] immobilized some quinones via covalent bonds on the surface of a pyrolytic graphite electrode. The immobilized quinones showed a catalytic activity, and the overpotential decreased at about 200 mV. However, the electrode lost its activity after a few cycles. Nevertheless, the report demonstrates the feasibility of electrode modification for a direct electron transfer to NADH.

Dopamine, with 1,2-quinone functionality, was reacted with poly(methacryloyl chloride) to form a polymer which was adsorbed on the surface of vitreous carbon electrodes in a very recent experiment described by Degrand and Miller [15]. The decrease in overvoltage was about the same as that reported by Tse and Kuwana. The stability was better, but the activity still decreased by 25% after five sweeps in the presence of NADH.

Brown et al. [16,17] deposited 9,10-phenanthrenequinone on a graphite electrode and studied the electrochemistry of the adsorbed layer. Similar studies have been reported from our laboratory [18,19]. Flavines, especially FAD, were found to adsorb strongly on the surface of a graphite electrode. The flavines were deposited from aqueous solutions. The electrodes retained their electrochemical activity for long periods when stored either in air or in solutions without any soluble flavines. The electrodes could be cycled repeatedly without much loss of activity. None of the adsorbed flavines showed any catalytic activity during the electron transfer from NADH.

This work reports on attempts to use adsorbed mediators from one of the other groups mentioned above, namely the phenazine methosulphate or its analogues. These mediators are known to be fast in homogeneous reactions, and their tricyclic ring system should facilitate a rapid electron transfer to the graphite.

EXPERIMENTAL

Spectrographic graphite (RW 003 Ringsdorf-Werke, GmbH) was mounted in a Teflon holder as described previously [18]. A circular electrode area with a diameter of 6.1 mm (0.29 cm²) contacted the solution. Measurements were made with either triangular sweep cyclic voltammetry with SCE as a reference electrode or with a rotating disc assembly (Tacussel EDI).

PMS was obtained from Serva (Cat. No. 32030), PES and NADH from Sigma Chemical Co. (Cat. Nos. N-8129 and P-4883 respectively)

All measurements were made in buffers made from 0.1 M pyrophosphate (pH 5.0, 6.0 and 9.0) or 0.1 M phosphate (pH 4.0, 7.0 and 8.0).

RESULTS AND DISCUSSION

Direct adsorption

Attempts were made to adsorb PMS directly on to the graphite electrode in the same way as previously reported for FAD, FMN and riboflavine (for formulae see

Fig. 1) [18,19]. The electrodes were dipped into $\sim 1 \text{ mM}$ solutions of PMS for various periods, washed and transferred to solutions without any PMS. The amount of PMS adsorbed was of the order of $10^{-10} \text{ mol cm}^{-2}$, i.e. only a small fraction of

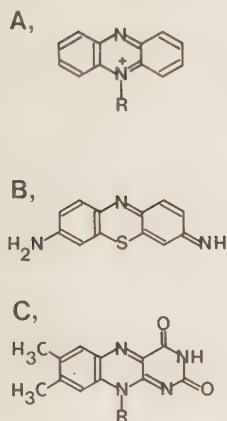


Fig. 1.(A) Formulae for the alkylphenazonium ion: R denotes -methyl for PMS and -ethyl for PES; (B) formulae for thionine; (C) formulae for the flavine sequence. R denotes -methyl for lumiflavine, -ribityl for riboflavine, -ribitylphosphate for FMN and -ribitylphosphate-adenine-nucleotide for FAD.

the coverage observed earlier for flavines ($2 \times 10^{-8} \text{ mol cm}^{-2}$). The PMS vanished easily, either after further washing or through dissolution with time. Varying the pH of the solutions had little or no effect. The conclusion must therefore be that the simple immobilization procedure by adsorption which occurred for the flavines does not work for PMS.

Voltage-facilitated immobilization

Albery et al. [20] reported on the modification of platinum and tin dioxide electrodes with thionine. They applied a positive voltage for 15 min (1–1.5 V vs. SCE) on electrodes immersed in a thionine solution. They estimated the organic coverage to be of the order of 20 monolayers. Such experiments with thionine were also made at this laboratory with platinum electrodes and the results of Albery et al. [20] were essentially confirmed. If the pH was increased from the value used by them (0.05 M H_2SO_4), to pH 4, the layer was immediately desorbed. The electrochemical activity was lost and did not reappear when the electrode was examined again in acid solution. The electrode could, however, be easily covered again in a thionine solution, using a positive voltage. Using graphite electrodes it was found that a coating was indeed obtained but that the voltammogram was irreproducible and distorted even in acid solution. The thionine layer was desorbed immediately in solutions of pH 4.

If the same procedure was repeated with PMS or PES the results were strikingly different. They were immobilized on graphite electrodes from a $\sim 1 \text{ mM}$ solution with $+1.1 \text{ V}$ vs. SCE applied for 15 min. A stable layer formed at pH 4, pH 7 and also at pH 9. (PMS is very unstable in acid solution.) The electrodes were washed and transferred to buffer solutions without any mediators.

The immobilization procedure did not work with PMS and platinum electrodes when tested at pH 4. All immobilizations were made in subdued light as PMS is also very light sensitive.

The amount of PMS which can be immobilized on a graphite surface, is independent of pH, and it increases with time at 1.1 V up to a constant level, which is reached after about 15 min. The amount remains constant even after 12 h immersion with a voltage applied. The amount corresponds to about $6 \times 10^{-9} \text{ mol cm}^{-2}$. There is an individual variation between different electrodes of about $\pm 20\%$. Electron microscopy gives the explanation for this, as it can be seen that the size of the flakes and pores of the material varies throughout the rod.

The coverage of PMS on a mole-for-mole basis is only a tenth of that observed for flavines using the same electrode material. The tricyclic ring systems are of about the same size, but the side chains of FAD especially are much larger, see Fig. 1.

There is a charge on one of the nitrogens of PMS, and this fact may be of importance for the differences in behaviour between PMS and the flavines which are neutral in the ring systems. The charge may prevent binding except to selected sites on the graphite. A graphite electrode which was first oxidized at $+1.1 \text{ V}$ vs. SCE in a buffer solution and then immersed in a PMS-containing solution retained some PMS upon transference to other solutions. The amount of PMS which was retained was much less than when the voltage was applied during immersion in a PMS solution. The experiments suggest that an oxidative modification of the graphite may be involved in the immobilization.

Electrochemistry of PMS

Electrochemical investigations of PMS in aqueous solutions are complicated by the low solubility of the reduced neutral form. On a Pt electrode, at pH 5–9, the oxidation–reduction is reported to be reversible, with an overall reaction mechanism involving two electrons and one proton [21].

Figure 2 shows cyclic voltammograms obtained at pH 7, with a graphite electrode in 1 mM PMS (Fig. 2a), in 1 mM PMS after 15 min at 1.1 V (Fig. 2b) and in a buffer with no PMS (Fig. 2c) respectively. The background for an untreated electrode is shown in Fig. 2a in broken lines. The first voltammogram (Fig. 2a) represents the behaviour with weakly adsorbed PMS, as well as PMS arriving at the electrode through diffusion during the sweep. The area of the oxidation peak is larger than that of the reduction peak. In fact, the size of the oxidation peak can be increased at will by stopping the sweep at a negative value. Reduced insoluble PMS precipitates on the electrode so that a higher concentration is obtained. A film can be observed visually after some time. In Fig. 2b the peak heights have increased

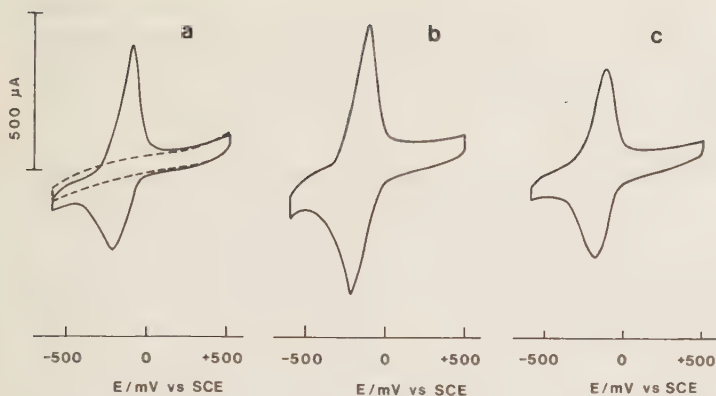


Fig. 2. (a) Cyclic voltammogram of 1 mM PMS, untreated graphite electrode. (---) Untreated graphite electrode in buffer with no PMS. (b) Cyclic voltammogram of 1 mM PMS, after 15 min at +1.1 V vs. SCE. (c) Cyclic voltammogram of immobilized PMS in buffer with no PMS, after 15 min at +1.1 V vs. SCE. 0.1 M phosphate buffer pH 7.0, sweep rate 100 mV s⁻¹.

further as soluble and immobilized and possibly also weakly adsorbed PMS contribute to the current. Figure 2c was obtained in a buffer after the electrode had been washed. It can be seen that the areas of the oxidation and reduction peaks are about the same as expected. The $E_{1/2}$ was found to be exactly the same for the immobilized and soluble PMS.

The $E_{1/2}$, which is -160 mV vs. SCE at pH 7, decreases by 30 mV/pH between pH 4 and 9 for the immobilized PMS in the same way as for the soluble PMS.

The peak voltage of the anodic peak increased slightly (20 mV) over a sweep interval from 25 mV s⁻¹ to 800 mV s⁻¹ at pH 7. The cathodic peak voltage decreased somewhat more (80 mV) under the same conditions. The peak separation is 45 mV for PMS at 25 mV s⁻¹, which contrasts with a peak separation of zero for an adsorbed molecule with a fast electron transfer. In this reaction a proton transfer through diffusion from solution has to be taken into account in addition to other non-idealities. The peak current increased linearly with the sweep rate from 25 mV s⁻¹ to at least 225 mV s⁻¹, but the slope of the plot is lower than theory for an adsorbed species. The criteria used to characterize diffusionless electrochemical systems are thus partly fulfilled by the PMS electrode.

PES-covered electrodes were also investigated with linear sweep voltammetry. The $E_{1/2}$ is -210 mV vs. SCE at pH 7. The cathodic peak potential decreased less with the sweep rate than for the PMS electrode. The coverage of the PES electrode was somewhat lower, 4×10^{-9} mol cm⁻². Some of the electrochemical differences can thus be ascribed to the lower coverage.

The lifetime depends on the operational conditions. In an unstirred buffer solution, pH 7, with continuous electrochemical cycling, the peak area of a PMS

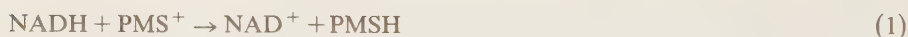
electrode decreased 80% over 15 h. The activity in a stirred buffer solution decreased 60% over 2 h. The decrease in activity is exponential rather than linear. The great effect of stirring indicates that the immobilized PMS is stripped from the electrode surface by dissolution.

Catalysis of the NADH oxidation

Figure 3 shows a cyclic voltammogram of 5 mM NADH in a phosphate buffer, pH 7, using an untreated graphite electrode. The wave is highly irreversible and it is distorted. The rising part of the peak changes with the condition of the electrode, as discussed by Moiroux and Elving [5]. Even an overshoot can sometimes be observed at about 450 mV.

Figure 4a shows the first sweep on a PMS electrode in a solution containing 5 mM NADH. It can be compared with the curve obtained with the PMS electrode in a buffer without any NADH (Fig. 4, broken line). A second sweep in the NADH solution taken immediately after the first (Fig. 4b) shows a decreased anodic current, whereas the cathodic parts coincide. If the solution was stirred or if some time was allowed to elapse, an additional sweep retraced curve a.

The two reactions responsible for the increased anodic current which peaks at -50mV are



and

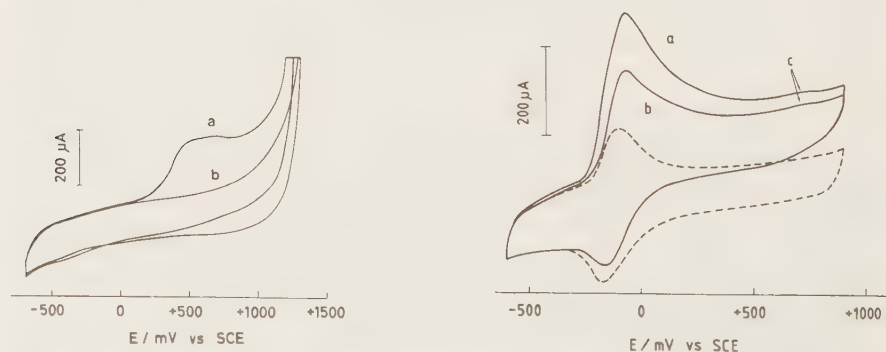
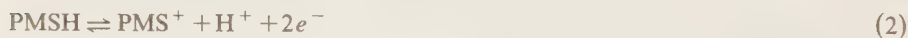
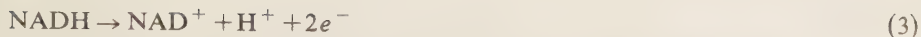


Fig. 3. (a) Cyclic voltammogram of 5 mM NADH, untreated graphite electrode. (b) Cyclic voltammogram with buffer only, untreated graphite electrode. 0.1 M phosphate buffer pH 7.0; sweep rate 50 mV s^{-1} .

Fig. 4. (---) Cyclic voltammogram of a PMS electrode in a buffer with no PMS. (a) First cyclic voltammogram of a PMS electrode in a buffer with 5 mM NADH. (b) Second cyclic voltammogram of a PMS electrode in a buffer with 0.1 M phosphate, pH 7.0; 50 mV s^{-1} . (c) The arrows indicate a slight shoulder at $+700\text{ mV}$ which increases as the deactivation of the electrode proceeds.

resulting in



The NADH diffuses up to the electrode surface where it reacts (eqn. 1) with the immobilized PMS, which in turn (eqn. 2) is reoxidized electrochemically. The potential at which this reoxidation occurs is determined by the electrochemical properties of the immobilized PMS. The overall reaction (eqn. 3) is an oxidation of NADH to NAD^+ with PMS acting as a catalyst.

The combined effect of NADH diffusion and reaction rates are further illustrated in Fig. 5 which was obtained with a rotating disc electrode. The adherence to theory of the rotating electrode was checked with a 4.0 mM ferrocyanide solution. A Levich line for NADH (Fig. 5) was calculated with a diffusion coefficient of $3.0 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ calculated from Fig. 2 in ref. 1.

Curve b in Fig. 5 shows the current variation with the square root of the rotation velocity for a freshly prepared PMS electrode. Curve c shows the result when a PMS electrode which had lost some of its activity was used. The rate of reaction thus depends not only on the mass transport but also on the amount of immobilized PMS on the electrode. The surface coverage of the two electrodes corresponded to 8×10^{-9} and $6 \times 10^{-9} \text{ mol PMS cm}^{-2}$ respectively. The currents (at 1450 rpm) were found to be proportional to the surface coverage, i.e. the amount of PMS on the electrode. It was also found that the current at this rotational speed was proportional to the concentration of NADH. The overall reaction rate in the limiting case (high rotational speed) is thus proportional to both the PMS and the NADH of eqn. (1). Equation (1) is thus rate determining, while eqn. (2) is much faster at an applied potential of -50 mV vs. SCE .

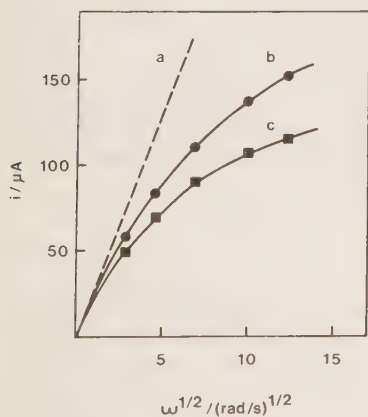


Fig. 5. Current variation with square root of rotating velocity. (a) Levich line for a very fast reaction; (b) result for a PMS electrode, coverage $8.0 \times 10^{-9} \text{ mol cm}^{-2}$; (c) result for a PMS electrode, coverage $6 \times 10^{-9} \text{ mol cm}^{-2}$, 1.7 mM NADH, 0.1 M phosphate buffer pH 7.0. Applied voltage = -50 mV vs. SCE .

TABLE I

$E_{1/2}$ vs. SCE at pH 7 of four modified electrode functionalities, as well as the peak potential, E_p , of the oxidation wave in the presence of NADH

	Quinone, ref. 11	Dopamine polymer, ref. 12	PMS, this work	PES, this work
$E_{1/2}/\text{mV}$	+160	+120	-160	-210
E_p/mV	+260	+215	-50	-100

A corresponding experiment at -50 mV with a naked graphite electrode in a NADH solution gave a current close to the background, i.e. zero on the scale used in Fig. 5.

PES electrodes were prepared and studied in the same way. The results were generally very similar, except that the oxidation peak potential became -100 mV vs. SCE in 5 mM NADH at pH 7. The overpotential for the NADH oxidation (eqn. 3) is thus reduced by 50 mV compared to that of the PMS electrode.

The direct NADH oxidation at the modified electrode surface is negligible for fresh electrodes. The arrows in Fig. 4c indicate a slight shoulder at +700 mV which increases as the deactivation of the electrode proceeds.

CONCLUSIONS

The $E_{1/2}$ values for the four modified electrodes which so far have been effective in a catalytic oxidation of NADH are shown in Table 1 (recalculated to pH 7). There has been a successive decrease in the overpotential due to the selection of electrode functionalities with more negative E^0 values. However, there is still a large gap left to the reversible potential [9] of the NADH/NAD⁺ couple (-560 mV vs. SCE). Nevertheless, the overpotential has been reduced by 550 mV compared to the naked graphite electrode and by at least 700 mV compared to oxidation at a Pt electrode (pH 7). The catalytic properties are thus extremely well demonstrated. The lifetime of the modified electrodes has also been increased, even if still more increase is necessary before any practical or technical application becomes feasible.

ACKNOWLEDGEMENT

The authors thank Prof. G. Johansson for valuable discussions concerning this work.

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Catalytic Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Graphite Electrodes Modified with Adsorbed Aromatics Containing Catechol Functionalities

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4-[2-(2-Naphthyl)vinyl]catechol (NSCH₂) and 4-[2-(9,10-ethanoanthracen-9-yl)vinyl]catechol (ASCH₂) were adsorbed on graphite electrodes. The naphthalene and the ethanoanthracene ring systems were used as anchors to the graphite. The catechol group is free to move out into the electrolyte solution. The electron transfer between the 1,2-hydroquinone functionality and the graphite was fast. The surface coverage was at most 9×10^{-9} mol cm⁻². The coenzyme reduced nicotinamide adenine dinucleotide (NADH) could be catalytically oxidized by the immobilized mediating groups. The overvoltage of the NADH oxidation decreased from 410 mV vs. SCE at the unmodified graphite electrode to 185 mV at the NSCH₂ covered electrode at pH 7.0. The reaction rate of the ASCH₂ covered electrode was lower than that of the NSCH₂ electrode. After 30 min of continuous electrochemical cycling of pH 7.0, 30% of the original coverage remained for the NSCH₂ electrode.

The coenzyme reduced nicotinamide adenine dinucleotide (NADH) can only be oxidized electrochemically at high overvoltages (1). The overvoltage at pH 7 is about 1.1 V at carbon (2) and 1.3 V at platinum electrodes (3). If the electrode has been pretreated and if the NADH concentration is at most 0.1 mM, the product at platinum electrodes is enzymatically active NAD⁺ (4). Electrodes modified so that NADH could be oxidized more easily should open up a new field of applications both in analysis and in biotechnology.

About 300 hydrogenases are known which are dependent on NADH or NADPH as redox transfer agents.

Oxidation of NADH to enzymatically active NAD⁺ in homogeneous solution can only be made with a few oxidizing agents, often called mediators.

Surface modifications have been tried as a means to reduce the overvoltage of the electrochemical oxidation. The strategy has been to immobilize functional groups with known mediating properties on the surface. Blaedel and Jenkins (5) could reduce the overpotential with 0.2 V to 0.45 V vs. SCE by an electrochemical pretreatment of the electrode. They presumed that hydroxyl, carbonyl, and quinone functionalities were produced. Tse and Kuwana (6) immobilized 1,2-hydroquinones via covalent bonds to the surface of pyrolytic graphite and showed that the functionalities had catalytic activity. The overpotential decreased further about 0.2 V compared to Blaedel and Jenkins. The work demonstrated the feasibility of making catalytic electrodes by the immobilization of mediating functional groups.

Degrand and Miller (7) made a polymer from poly(methacryloyl chloride) and dopamine which adsorbed on the surface of vitreous carbon. The decrease in overvoltage was about 0.25 V. The surface coverage and lifetime were improved compared to the electrode made by Tse and Kuwana.

It has been observed that a number of polycyclic aromatic compounds adsorb strongly to graphite surfaces. Anson and co-workers studied the electrochemistry of 9,10-phenanthrenequinone (8, 9) and [1-(9-phenanthrene)-2-(4-pyridine)ethene] (10) adsorbed on graphite. Laviron et al.

(11) reported on the electrochemistry of benzo[c]cinnoline adsorbed on graphite. Coenzymes from the flavine group were studied in our laboratory (12, 13). The flavines adsorbed strongly to graphite, and the electron transfer between the functional group and the graphite was quite rapid.

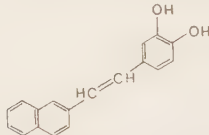
As it is known that some 1,2-quinones can be mediators in homogeneous solution there is a possibility that the adsorbed 1,2-quinones should be able to mediate electron transfer from NADH. No such effect could be seen for the 9,10-phenanthrenequinone (6) or for a greater number of condensed aromatic quinones studied preliminarily in our laboratory. The position of the adsorbed quinone functionality, which probably lies flat along the surface, seems to be unfavorable for electron exchange with NADH. Utilization of this mediator seems to be possible only if the functional group can be raised somewhat from the surface. The success reported by other types of immobilization (6, 7) supports this belief.

This paper reports on the adsorption of two types of compounds which have a 1,2-hydroquinone functionality in a side ring connected to a larger aromatic ring system. The larger aromatic ring systems anchor the compounds to the graphite surface and the smaller catechol acts as a mediator. The anchoring aromatic ring also provides a fast electron exchange between the graphite electrode and the adsorbed functionality.

A voltage facilitated immobilization of phenazine methosulfate mediator on graphite has simultaneously been made in our laboratory (14). These electrodes also showed catalytic activity for the NADH oxidation.

EXPERIMENTAL SECTION

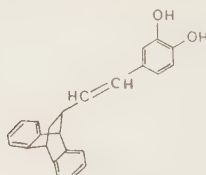
Syntheses. 4-[2-(2-Naphthyl)vinyl]catechol (I) was synthesized



from 2-naphthalene aldehyde and 3,4-dimethoxybenzyl alcohol. The dimethoxybenzyl alcohol was treated with PCl_5 in dry ether at 0°C according to Carman and Shaw (15) to produce the acid chloride. The triphenylphosphonium salt was made by boiling the chloride with triphenylphosphine in dry toluene at 110°C for 48 h (16). The formed precipitate was washed with dry cold ether. A Wittig reaction was made between a slight excess of the phosphonium salt and 2-naphthalene aldehyde dissolved in methylene chloride by addition of 50% aqueous sodium hydroxide. The mixture was agitated vigorously for 20 min (17). The organic layer was separated and the solvent was evaporated.

The product was purified by passing a solution of the product in petroleum ether-ethyl acetate 50:50 through a column of silica gel 60 as described by Still et al. (18). Only a small amount of *cis*-stilbene is formed due to steric hindrance. Furthermore the *cis* and *trans* isomers elute in separate fractions from the column. Only the *trans* form was collected. The ether groups were cleaved by treatment with trimethylsilyl chloride and sodium iodide in dry acetonitrile (19). The necessary reaction time was considerably longer than the one reported (19), probably due to steric hindrance between the ortho silyl ethers formed prior to hydrolysis.

The 4-[2-(9,10-ethanoanthracen-9-yl)vinyl]catechol (II) was



made from anthracene, acrolein, and dimethoxybenzyl alcohol.

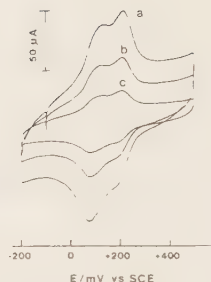


Figure 1. Cyclic voltammogram of NSCH_2 adsorbed on graphite in 0.1 M phosphate buffer, pH 7.0, surface coverage $0.7 \times 10^{-9}\text{ mol cm}^{-2}$, starting potential -100 mV vs SCE , and sweep rate (a) 200, (b) 100, and (c) 50 mV s^{-1} .

In the first step 9,10-ethanoanthracene carboxaldehyde was synthesized by a Diels-Alder reaction between anthracene and acrolein in toluene during 16 h at 150°C in a steel bomb (20). The reaction mixture was filtered and the solvent of the filtrate was evaporated. The aldehyde was dissolved in methanol and any residual anthracene was removed by filtration. The aldehyde monohydrate was formed by adding water and the precipitate was filtered off. The monohydrate was transformed back to the aldehyde at 100°C under vacuum. The steps of the synthesis and the purification were the same as described above for the compound I. The products, I and II, were identified by IR, NMR, mass spectrometry, and elemental analysis.

Measurements. The electrodes were spectrographic graphite, 6.1 mm o.d. (RW 003 Ringsdorf-Werke, GMBH). They were polished with emery paper (Tufbak Durite Waterproof Paper Closecote 600-A Type 1) put on a rotating disk polishing machine. The polishing was performed under a continuous flow of deionized water for 5 min. After that, the electrodes were dried in air. Before mounting the electrodes in the holder for the electrochemical experiment, a rubber tube (6.0 mm i.d., isoversinic, Kebo Grave) was slipped over in order to avoid electrical contact between the sides of the electrodes and the solution. This was done from the opposite end of the polished surface in order to avoid affecting the surface mechanically or chemically. The edge of the tube was put just in line with the electrode surface.

The electrochemical surface area was identical with the geometric one when measured in a 2 mM ferrocyanide solution using both a stationary and a rotating disk arrangement. The rubber tubing as well as the sides of the electrodes were quite hydrophobic and no creeping of solution between the electrode and the rubber tubing was detected. Measurements were made with either triangular sweep voltammetry or with a rotating disk assembly (Tacussel EDI) using the SCE as a reference electrode.

All measurements were made in buffers made from 0.1 M pyrophosphate (pH 5.0, 6.0, and 9.0) or 0.1 M phosphate (pH 2.0, 3.0, 4.0, 7.0, 8.0, and 11.0).

The synthesized products were first dissolved in methanol-ethanol 80:20. An equal volume of aqueous 0.1 M hydrochloric acid was added when the dissolution was complete. The resulting solution was about 0.5 mM . The graphite rods were dipped into this solution under slow agitation for 3–10 min depending on the desired coverage.

The purity of the synthesized products was checked by HPLC on Nucleosil-CN, $-\text{NH}_2$, and $-\text{C}_8$, Hypersil ODS-C18 (all $5\text{ }\mu\text{m}$), and μ -Porasil-SiOH ($10\text{ }\mu\text{m}$) columns using several different methanol-ethanol mixtures as well as acetonitrile as solvents.

RESULTS AND DISCUSSION

Electrochemistry of Adsorbed NSCH_2 . Figure 1 shows cyclic voltammograms of the NSCH_2 covered electrode, obtained in a buffer solution with no soluble NSCH_2 present. The adsorption of NSCH_2 had taken place during 3 min from the solution specified in the Experimental Section. After that the electrode had been washed thoroughly with deionized water. The figure demonstrates the properties of the adsorbed

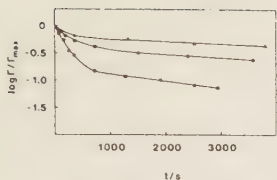


Figure 2. Desorption of NSCH₂ vs. time in an unstirred solution: (▲) pH 5.0; (●) pH 7.0; (■) pH 9.0 and 11.0.

layer. Two peaks are observed. The peak current is linearly proportional to the sweep rate over a range of 25–600 mV s⁻¹ indicating a diffusionless system. The separations between the first anodic and the corresponding cathodic peaks are 40, 50, and 60 mV for the three sweep rates. The corresponding peak separations for the second peaks are 20, 25, and 35 mV. For a fully reversible reaction in which no reactants from the solution take place, the peak separation should be zero (20). The relatively small peak separations indicate fairly fast electrochemical reaction rates (21). If the coverage of adsorbed species is decreased even smaller peak separations are obtained (22). The apparent rate constants, calculated according to these references, were 3–6 s⁻¹, about the same for both steps. The two peaks on both the anodic and cathodic sweeps indicate that the reactions occur in two one-electron steps. The intermediate should be the semiquinone, resonance stabilized by the double bond to the naphthalene ring. A further indication that the reaction proceeds with a one-electron mechanism is that the peak widths at half-height are 90–95 mV (theory 90.6/n mV (23)). A plot of the peak current over the charge vs. the sweep rate also gives evidence of two separate electron steps, as *n* values close to 1 are obtained from the slopes (8). The two electrochemical peaks cannot be caused by two different species as these would have been separated by at least one of the HPLC columns mentioned in the Experimental Section.

The electron transport can take place through different mechanisms. Electrons can be transferred through the double bond resonance structure between the catechol and the anchoring naphthalene ring. The catechol is free to interact directly with the surface so that the electron transfer might also take place through a flip-flop movement. Even an electron tunneling mechanism seems probable.

A plot of *E*_{1/2} vs. pH from pH 1 to 9 gives a slope of 59.4 mV/pH unit at 25.0 °C. It can therefore be concluded that an equal number of electrons and protons is involved in the two electrode reactions. The *E*_{1/2} at pH 7.0 for the two waves were +95 and 190 mV, respectively.

The amount of NSCH₂ on the electrode increased asymptotically with the time spent in the adsorption solution. There was a leveling off after about 60 min. The highest coverage was around 9 × 10⁻⁹ mol cm⁻². The shape of the cyclic voltammograms changes with coverage. The two peaks merge at high coverage and the separation between the anodic and cathodic peaks increases (23).

Stability. Figure 2 shows the rate of desorption of NSCH₂ from the graphite at four different pH values. The measurements were made in unstirred solutions with a continuous electrochemical scanning back and forth. There is a marked increase of the desorption rate with pH. The p*K*_a for catechol itself is 9.85 and the solubility of the reduced form will therefore be higher at higher pH due to deprotonation. No change in stability is observed between pH 9 and 11. The rate of desorption increases somewhat when the solution is stirred. The electrode is stable when stored in air.

Catalytic Oxidation of NADH at the NSCH₂ Electrode. Cyclic Voltammetry. Figure 3, curve a, shows a cyclic

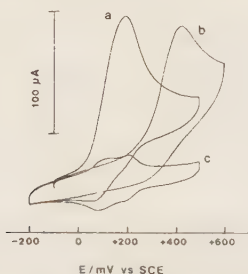
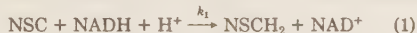


Figure 3. Catalytic oxidation of NADH mediated by adsorbed NSCH₂ (curve a), 2.3 mM NADH, pH 7.0, sweep rate 50 mV s⁻¹. Curve b shows oxidation at a naked graphite electrode in the same solution. Curve c shows NSCH₂ modified electrode in a buffer without NADH. Starting potential was -100 mV vs. SCE.

voltammogram obtained with the NSCH₂ covered electrode in a solution which was 2.3 mM in NADH. It can be seen that there is a great increase in the anodic current compared to a scan in a buffer without NADH (curve c). The reason is that NADH diffuses up to the electrode surface and reduces the NSC to NSCH₂. As NSCH₂ is regenerated by NADH during the sweep, there will be an increase in the current. For the same reason the cathodic current is smaller in the presence of NADH as NSCH₂ is produced simultaneously from both an electrochemical and a chemical step. The overall reaction thus proceeds as shown in eq 1–3. The rate-determining step



is given in eq 1 with a rate constant *k*₁. Thus the rate of the reaction in eq 2 can be considered fast compared to the one in eq 1. The overall oxidation of NADH by the electrode is given in eq 3.

The catalytic effect can be seen directly when curve a is compared with curve b in Figure 3. Curve b is obtained with an untreated graphite electrode. The decrease in overvoltage is 225 mV at pH 7.0 and 315 mV at pH 9.0.

Succeeding scans with stirring between them result in only minor decreases of the peak height of curve a. The decrease follows the course given by Figure 2 which indicates that a repeated recycling can take place without loss of activity from other sources than desorption of NSCH₂. The peak current was proportional to the NADH concentration in the range tested 0.7–7 mM. Andrieux and Saveant (24) derived a relation between the peak current and the concentration for the case when *k*₁ in eq 1 is infinitely fast.

$$i_p = 0.496nFAD_R^{1/2}v^{1/2}C_R^*(nF/RT)^{1/2} \quad (4)$$

The symbols have their usual significances. Low values of *k*₁ result in lower values of the constant (0.496). This constant was found to be 0.30 for a NSCH₂ electrode with a coverage of Γ = 1.3 × 10⁻⁹ mol cm⁻². The rate constant *k*₁ in eq 1 associated with this value could then be calculated to ~2 × 10⁶ M⁻¹ s⁻¹.

Rotating Disk Experiments. Experimental results with the NSCH₂ covered electrode mounted in a rotating disk assembly are shown in Figure 4. A Levich line, assuming a very fast chemical reaction, was calculated (curve a) by using a diffusion coefficient of 2.4 × 10⁻⁶ cm² s⁻¹ (25) for NADH.

The currents obtained in NADH solutions are shown by curves b–d. The potential was kept at +275 mV vs. SCE, i.e., just on the plateau obtained when the voltage was scanned

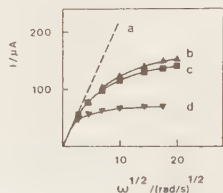


Figure 4. Anodic oxidation of NADH on a rotated disk graphite-NSC electrode. Curve a shows the theoretical line for a very fast reaction. Experimentally obtained curves are shown for a surface coverage of (b) 1.3×10^{-4} , (c) 1.5×10^{-4} , and (d) 0.5×10^{-4} mol cm $^{-2}$. Applied voltage was +275 mV vs. SCE, pH 7.0. Concentrations for a, b, and d were 1.90 mM and for c was 1.76 mM NADH.

slowly during rotation. The rate of rotation was increased stepwise. Following each step the current stabilized in a few seconds. When the rotation rate was returned to the original slower value, the current had decreased according to the desorption in Figure 2. An unmodified electrode gave no oxidation current at this voltage. The NADH concentrations in b and c were 1.90 mM and in d, 1.76 mM. Curve b was obtained with an electrode with a surface coverage of 1.3×10^{-4} mol cm $^{-2}$. The coverages for curves c and d were 1.5 and 0.5×10^{-4} mol cm $^{-2}$, respectively.

As seen in Figure 4 the current reaches a maximum level as the rotation speed is increased. This is expected in a rotating disk experiment, when the current is completely under kinetic control. Andrieux et al. (26) derived the following equation for surface modified catalytic electrodes when the current has reached its maximum value.

$$i = nFAk_1\Gamma_{\text{ox}}C_R^* \quad (5)$$

The symbols have their usual significance and k_1 is the rate constant of eq 1. At the potential used the rate of the electrochemical reaction, eq 2, can be considered infinite and the reaction in eq 1 is the rate-determining step. In this way the curves shown in Figure 4 give k_1 values of 1.2, 1.0, and 1.4×10^6 M $^{-1}$ s $^{-1}$ which agree well with the one evaluated from the cyclic voltammetrical experiments.

Electrochemistry of Adsorbed ASCH₂. Cyclic voltammograms of ASCH₂ adsorbed on graphite electrodes were generally very similar to those of the adsorbed NSCH₂ except that the electrochemical reaction rate was somewhat smaller. The apparent rate constant was about 0.8 s $^{-1}$. The $E_{1/2}$ and its pH dependence were the same and there were also two peaks indicating separate one-electron steps.

The intramolecular conduction of the compound II should be smaller than that of compound I, and it was found that the electrochemical rate is at least 3 times slower than that for the naphthalene. The original coverage was about the same but the desorption was faster. The desorption was in fact too fast to make experiments with a rotating disk electrode with a constant and well-defined coverage.

Catalytic Oxidation of NADH on the ASCH₂ Electrode. Figure 5 shows a cyclic voltammogram obtained with ASCH₂ adsorbed on a graphite electrode. Curve c shows the voltammogram in a buffer without other reagents. The surface coverage was 0.7×10^{-4} mol cm $^{-2}$. In the presence of 2.4 mM NADH a catalytic oxidation occurs at (a) in the upper curve. At more anodic voltages, (b) in Figure 5, NADH is oxidized directly without involvement of the attached mediating groups.

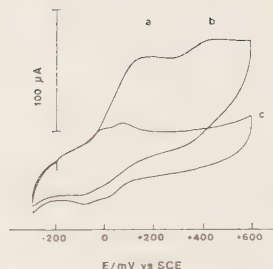


Figure 5. Catalytic oxidation of NADH mediated by adsorbed ASCH₂ (a) as well as direct oxidation of NADH (b). Surface coverage was 0.7×10^{-4} mol cm $^{-2}$ with 2.4 mM NADH, pH 9.0, and sweep rate 50 mV s $^{-1}$. Curve c was obtained under the same conditions except that no NADH was present. Starting potential was -200 mV vs. SCE.

A comparison between the catalytic oxidation of the NSCH₂ and the ASCH₂ electrodes (Figures 3 and 5) shows that the reaction between NADH and the mediating group is much slower for the ASCH₂ electrode. The overvoltage was decreased less effectively than on the NSCH₂ electrode. As a matter of fact the ASCH₂ species was synthesized in the belief that the rate of the chemical step would be favored by the more elevated position of the catechol group.

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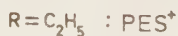
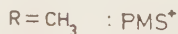
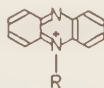
CATALYTIC OXIDATION OF REDUCED NICOTINAMIDE ADENINE
DINUCLEOTIDE BY GRAPHITE ELECTRODES MODIFIED WITH ADSORBED
PHENOXAZINIUM SALTS

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Summary

The reduced nicotinamide adenine dinucleotide coenzymes, NADH and NADPH, were oxidized electrocatalytically at graphite electrodes modified by adsorption of 7-dimethyl-amino-1,2-benzophenoxazinium (MB^+ , Meldola's Blue). The first step is a chemical oxidation of the coenzymes by the adsorbed MB^+ (rate constant $6 \cdot 10^3 \text{ M}^{-1} \text{ s}^{-1}$). The second step, which is much faster, is an electrochemical reoxidation of the mediator. Since the reoxidation takes place at a potential mainly determined by the E_1 of the adsorbed MB^+ ($E_1 = -170 \text{ mV vs SCE at pH 7.0}$) the overvoltage is 500 mV less than direct oxidation of the coenzymes at unmodified graphite electrodes. The electrocatalytic activity increased with coverage up to $3 \cdot 10^{-10} \text{ mol cm}^{-2}$ and remained essentially constant thereafter, indicating formation of multiple layers. About 15% of the adsorbed mediator was lost after two hours at pH 6, independently of the oxidation state.

Procedures for immobilizing reagents on electrodes now include covalent bonding between reagents and electrode materials, coating the electrode with polymers and adsorption of reagents containing functional groups (1). Electrocatalytic properties of the modified electrodes have been demonstrated for several reactions which normally require a high overvoltage. The oxidation of the nicotinamide coenzymes (NADH and NADPH) has attracted special interest due to the important practical application of a successful electrocatalysis. The electron transfer between these coenzymes and other redox molecules is normally very slow except for a few classes of redox compounds (mediators). The strategy followed in electrode modification research has been to produce or immobilize mediator functionalities on a surface of glassy carbon or graphite. 1,2-quinone functionalities have been attached through covalent bonding (2,3), through adsorption of a polymer (4) and through adsorption of an aromatic ring system with a side chain containing a catechol ring (5,6). Immobilized alkylphenazinium ions (PMS^+ and PES^+) have also shown electrocatalytic activity for the oxidation of coenzymes (7).



There was no electrocatalytic activity for quinones adsorbed directly on the electrode surface. The functional group has to be elevated in order to interact with the coenzyme (2,5). Such a strategy was not necessary with the alkylphenazinium ions.

The most important factors to be considered in evaluating the success of such a modified electrode are 1) The decrease in overvoltage compared to an unmodified electrode; 2) The life-time of the modified electrode; 3) The rate of reaction with the coenzyme and the rate of electron transfer between the electrode material and the mediating group.

The low stability of the electrodes has so far been the main problem. The deactivation of covalently bound 1,2-quinones were recently studied by Ueda et al (3) who found that the loss of attached mediator molecules played a minor role compared to other deactivation reactions. The mediator group was inactivated either by polymerization or by reaction with oxidation products of the coenzyme.

We decided to study the stability and other properties of a polyheterocyclic mediator as a continuation of our earlier work (7). The 7-dimethylamino-1,2-benzo-phenoxazinium ion (MB^+ , Meldola's Blue, formula see eq. 1) contains one ring more than PMS^+ and a stronger adsorption to graphite is therefore expected. The losses of activity due to desorption should therefore decrease. The properties of soluble MB^+ have earlier been compared to those of PMS^+ (8,9). The chemical stability of MB^+ seems to be better, and it is much less sensitive to light. The rate of reaction with the coenzyme seems to be about the same as that of PMS^+ .

Experimental

Rods of spectrographic graphite (RWO, Ringsdorff-Werke, GmbH) were pressed into a Teflon holder so that a flat circular surface (diam 3.05 mm) contacted the solution. The electrodes were polished on wet fine emery paper and washed thoroughly in deionized water. Potentiostatic triangular sweep voltammetry was made with a stationary electrode in quiet solutions using SCE as a reference electrode and Pt wire as a counter electrode. Rotating disk electrode (Tacussel, EDI) experiments were made at 25°C, either at a stationary potential, or with a very slow (3 mV s^{-1}) anodic sweep.

The amount of mediator attached to the surface was evaluated from the integrated peak of a linear scan, corrected for the background current. The same peak areas were obtained in the anodic and the cathodic directions.

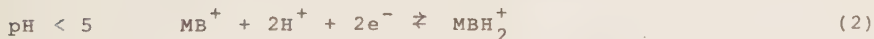
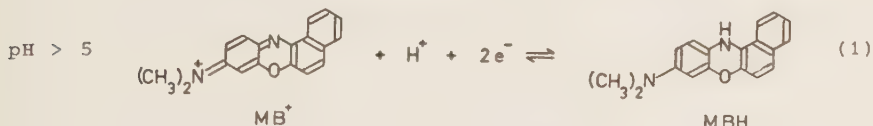
0.1 M phosphate buffers were used at pH 2-10, and 0.1 M HCl at pH 1.1. All measurements, except the spectrophotometric studies, were made in solutions deaerated by nitrogen bubbling. 7-dimethylamino-1,2-benzophenoxazinium ion (zinc-chloride salt) (MB^+) was obtained from Sigma Chemical Co (cat. no. D-8142). It should be observed that the numbering used by Sigma starts from the nitrogen (8-dimethylamino-2,3-benzophenoxazinium ion). NADH (1,4-dihydronicotinamide adenine dinucleotide) and NADPH (1,4-dihydronicotinamide adenine dinucleotide phosphate) were also obtained from Sigma, cat. no. N-8129 and N-7505 respectively.

Modification of electrodes was made by rotating ($\omega = 99.2 \text{ rad s}^{-1}$) a graphite electrode in a buffer solution containing 10^{-4} M MB^+ for 30 - 60 s depending on the desired coverage. The electrode was then washed thoroughly with deionized water.

Results and Discussion

Electrochemistry of the adsorbed mediator

A modified electrode, with less than monolayer coverage of MB^+ , was investigated electrochemically in pure phosphate buffers. The mediator can be repeatedly oxidized and reduced (Fig. 1 a) with an $E_{1/2}$ of $-170 \pm 5 \text{ mV vs SCE (pH 7.0)}$. A plot of $E_{1/2}$ vs pH gives one straight line from pH 2.0 to 5.0 with a slope of 60 mV/pH-unit and another straight line from pH 5.0 to 10.0 with a slope of 30 mV/pH-unit. The overall reactions are



The fact that the peak width at half height (70 mV at pH 1) is less than 90 mV gives support for a two electron process (10).

The peak separation, ΔE_p , depends on pH and on the sweep rate. It is close to zero at pH 1.1 (Fig. 2, curve a) and it increases symmetrically around $E_{1/2}$ when the sweep rate increases (Fig. 3). At first the pH dependence of ΔE_p may seem complex (Fig. 2) but it can be rationalized if the buffer capacity is taken into

account. The theory for surface attached redox compounds predicts a zero peak separation for a reversible process. It is tacitly assumed that the proton transfer can take place without any depletion of the surrounding solution (11). The assumption holds only if the proton concentration is high compared to the amount of adsorbed reactant. If a small pH change occurs more protons can be made available through deprotonization of buffer ions provided that both the buffer capacity and the proton exchange rate are high. The buffer capacity is especially low at pH 5.0 and consequently ΔE_p is larger than for any other pH (Fig. 2, curve c). Curves d and f illustrates the effect of an increased buffer capacity at the same pH, although the constants will be affected by changes in ionic strength (compare curve e). The fact that ΔE_p at a given pH increases with coverage gives further credence to the given explanation. We have not been able to find any quantitative treatment of these effects in the literature.

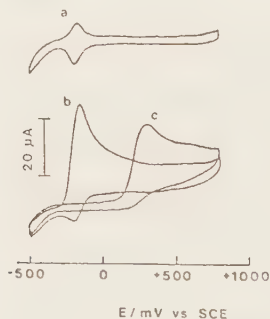


Fig. 1. Catalytic oxidation of NADPH mediated by adsorbed MB^+ (curve b), 3.1 mM NADPH, pH 7.0, sweep rate $50 \text{ mV} \cdot \text{s}^{-1}$.

Curve c shows oxidation at a naked graphite electrode in the same solution. Curve a shows MB^+ modified electrode ($\Gamma = 1.8 \cdot 10^{-10} \text{ mol} \cdot \text{cm}^{-2}$) in a buffer without NADPH. Starting potential was -500 mV .

The electrochemical reaction rate constant cannot be evaluated unless the effects of proton transfer can be taken into account. It can be concluded, however, that it is much faster than the chemical reaction.

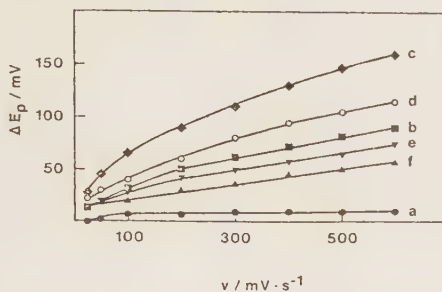


Fig. 2. Variation of ΔE_p with sweep rate in various buffers ($\Gamma = 1.6 \cdot 10^{-9} \text{ mol cm}^{-2}$)

curve	components	pH	β	I/M
a	0.1 M HCl	1.1	0.23	0.1
b	0.1 M phosphate	3	0.027	0.093
c	0.1 M phosphate	5	0.002	0.102
d	0.1 M phosphate	7	0.054	0.176
e	0.1 M phosphate 0.5 M NaNO_3	7	0.054	0.676
f	0.25 M phosphate	7	0.136	0.441

β = buffer capacity

I = ionic strength

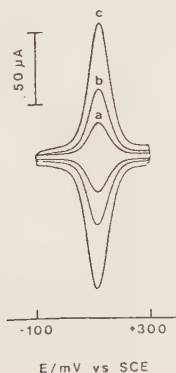


Fig. 3. Cyclic voltammograms of MB^+ in 0.1 M HCl at various sweep rates ($\Gamma = 2.2 \cdot 10^{-9} \text{ mole} \cdot \text{cm}^{-2}$) a = $50 \text{ mV} \cdot \text{s}^{-1}$; b = $100 \text{ mV} \cdot \text{s}^{-1}$;

Stability of MB⁺

Spectra of oxidized MB⁺, dissolved in buffer solutions, had peak absorbances at 220, 270, 315, and 565 nm (pH 7.0). The decrease in absorbance at 565 nm was recorded as a function of time.

The logarithm of the absorbance was found to decrease linearly with time at pH ≥ 7 . The linearity proves that MB⁺ decomposes according to a first order reaction. The slopes of the lines, which are given in Table I, decrease about 10 times when pH decreases one unit.

TABLE I

Spectrophotometric determination of the decomposition rate for MB⁺ in 0.1 M phosphate buffers at different pH.

($\lambda = 565$ nm) $t_{\frac{1}{2}}$ = half life time

pH	$\frac{d \log A}{dt} / h^{-1}$	$t_{\frac{1}{2}} / h$
6	-	> 100
7	-0.003	100
8	-0.02	15
9	-0.12	2.5
10	-1.4	0.2

The stability of MB⁺ when adsorbed on graphite was studied with cyclic voltammetry on stationary electrodes in deaerated neutral or acidic buffers. A plot of the logarithm of the amount of substance versus time yielded graphs with two linear segments. The first segment represents a more rapid loss than the second. Obviously several reactions occur simultaneously and a detailed analysis is impossible unless a much more thorough investigation is made. Some tentative conclusions can be drawn from the curves, however. The stability is very good (99% remained after two hours) at pH 1.1 and pH 3.0. The compound itself is stable

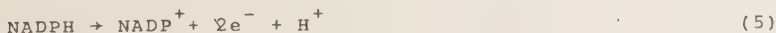
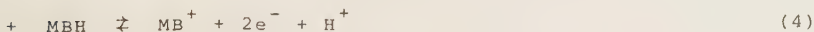
in acid solutions and the desorption of MB^+ and MBH_2^+ seems to be small in spite of the charges. The oxidation state had some influence at all pH, the lowest stability was observed when the electrode was cycled continuously. This may indicate that the intermediate radical decomposes faster than the oxidized or reduced form. 75, 85, and 65% of the substance remained on the electrode after two hours of continuous cycling at pH 5, 6, and 7. The losses of activity is thus much faster than the decomposition of the soluble compound.

A new redox couple, $E_{1/2} = -475$ mV vs SCE, can be seen when the electrode is cycled continuously at pH 7.0. It increases with time but it is much smaller than the decrease of the main peak. Storage in oxidized or reduced form at pH 7.0 results in traces of the compound. The peak could not be detected at lower pH. A compound with these properties could not be adsorbed from completely decomposed solutions of MB^+ .

Electrocatalytic oxidations of NADH and NADPH

The electrocatalysis of the MB^+ -modified electrodes is demonstrated in Figure 1. Scan a is a cyclic voltammogram of the modified electrode in a pure buffer solution and scan b in a solution containing NADPH. For comparison an unmodified electrode is scanned in a NADPH-containing solution, (scan c). It can be seen that the overvoltage is decreased 500 mV by the adsorbed mediator.

The NADPH diffuses up to the electrode surface where it reduces adsorbed MB^+ to MBH which in turn is reoxidized electrochemically.



Scan b gives a much larger anodic current than scan c (Fig. 1) because eq (3) and eq (4) take place simultaneously. MBH is regenerated by NADPH during the sweep resulting in a much larger anodic current than that required to oxidize the mediator itself. The oxidation potential is mainly determined by the E_1 of the mediator (eq 4), but also to some degree by the diffusion coefficients of the coenzyme, the coverage, the sweep rate and the rate constant of the chemical step (12).

The coenzyme NADH can also be oxidized electrocatalytically in the same way. A voltammogram with NADH becomes almost identical to Figure 1 under the same conditions.

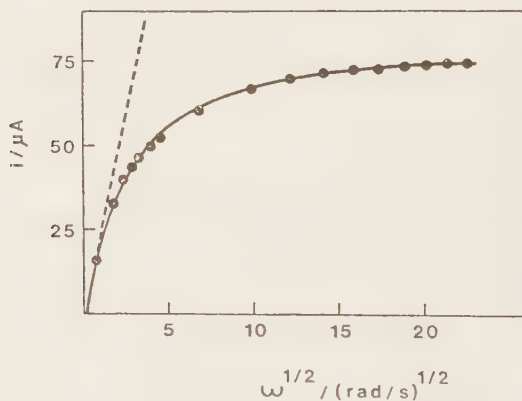


Fig. 4. Anodic oxidation of NADH on a rotated disk graphite-MB⁺ electrode. The broken line shows the theoretical response for a very fast reaction. An experimentally obtained curve is shown for a surface coverage of $4.4 \cdot 10^{-10} \text{ mol} \cdot \text{cm}^{-2}$. Applied voltage was 0 mV vs SCE, pH 7.0. Concentration of NADH was $7 \cdot 10^{-3} \text{ mol} \cdot \text{l}^{-1}$.

The mass transfer can be calculated very precisely for rotating disk electrodes. The maximum current for a very fast reaction was calculated for different rotational speeds (Fig. 4, broken line) assuming a diffusion coefficient of NADH of $2.4 \cdot 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ (13), see Appendix, eq 1. The actual currents were recorded when the modified electrode was kept at 0 mV vs SCE in NADH-containing buffers (Fig. 4, solid line). The current levels off towards a limiting value at high rotational speeds due to the limited rate of eq 3. The mass transfer becomes fast compared to the rate of the chemical reaction so that the current will be a measure of the rate of the chemical reaction. The rate of the electrochemical step will be much higher again at this potential so that at high rotational speeds the current is almost completely due to the electrocatalytic current. The current will be less than 1 μA for an unmodified electrode under these conditions.

The catalytic activity increases when the amount of substance, Γ , is increased (Fig. 5). The measurements were made at high rotational speed, the broken line at the top indicates the current expected if all NADH transported up to the electrode had been oxidized. It can be seen that the catalytic current levels off when Γ is increased and that this is not due to mass transfer limitation. The most likely explanation is that the mediator forms double or multiple layers on the electrode and that only the top layer is catalytically active.

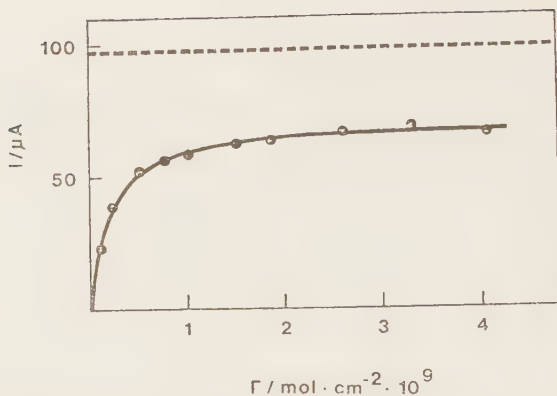


Fig. 5. The catalytic current of a rotating MB^+ -modified graphite electrode as a function of coverage. The broken line shows the theoretical response with an infinitely fast chemical reaction. Concentration of NADH 2.91 mM, pH 7.0. Angular velocity 99 rad s^{-1} .

Electron transfer must take place readily from the top MB^+ -layer through the stacked mediator molecules into the graphite. Up to $3 \cdot 10^{-8} \text{ mol MB}^+ \text{ cm}^{-2}$ have been adsorbed without any indication of current blocking. A blocking should have resulted in a negative slope of the curve in Figure 5. In fact the solid line has a slightly positive slope. This indicates either that stacking starts before the surface has been completely covered or that the stacking is irregular so that additional catalytic sites become exposed to the coenzyme. The mediator might also penetrate into the pores so that a larger catalytic surface is indeed obtained. The rate of reaction will be low in the pores due to slow coenzyme mass transfer.

Using the first model, i.e. that stacking starts before the coverage is complete, Γ^0 , the effective coverage can be determined by extrapolation. A line is drawn through the two initial points in Figure 5 and another through the almost horizontal

line. The intersection corresponds to $\Gamma^0 = 3 \cdot 10^{-10} \text{ mol cm}^{-2}$. The rate constant k_1 in eq 3 can be calculated from the measurements on a partly covered surface using eq 13 and it was found to be $6 \cdot 10^3 \text{ M}^{-1} \text{ s}^{-1}$.

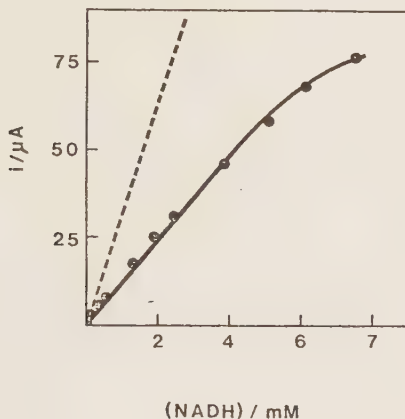
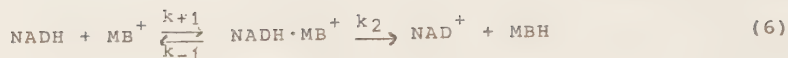


Fig. 6. The limiting anodic current on a rotated disk graphite - MB^+ electrode as a function of NADH concentration. The broken line shows the theoretical line for a very fast reaction. Coverage of MB^+ was $4.4 \cdot 10^{-10} \text{ mole} \cdot \text{cm}^{-2}$. Applied voltage 0 mV vs SCE, pH 7.0. Angular velocity $99 \text{ rad} \cdot \text{s}^{-1}$

The catalytic current increases linearly with increasing co-enzyme concentration (Fig. 6) up to a certain value depending on the surface coverage and the rotational speed. As the NADH concentration increases an increased fraction of the available mediator molecules will be present in the form of a mediator-coenzyme complex. Eq (3) can therefore be given in a more complete form



$$k_1 = (k_{-1} + k_2) / k_{+1} \quad (7)$$

where k_1 is the overall rate constant of eq 3.

Properties of MB⁺-modified electrodes compared to other modifications

MB⁺-modified electrodes have several advantages over electrodes modified by attachment of PMS⁺ or PES⁺ (7). The rate of desorption is much lower due to the larger ring system of MB⁺. It was not, however, possible to determine to what extent desorption contributes to the observed losses of substance from the electrode surface. PMS⁺ and PES⁺ decompose by dealkylation of the nitrogen. There is no corresponding side reaction with MB⁺. The overall stability of MB⁺-electrodes is much superior to PMS⁺- or PES⁺-electrodes. PMS⁺ and PES⁺ are unstable in both alkaline and acid solutions (14). They are very light sensitive and sensitive to oxygen in alkaline solutions. In fact the more detailed study of the MB⁺-electrode has been made possible by its greater stability.

The maximum catalytic activity of MB⁺-electrodes is obtained when the surface is completely covered with mediator. The size of the rate constant k_1 therefore sets an upper limit to the reaction rate at a given electrode surface. The rate can only be increased in so far as compounds with a higher reactivity with the coenzymes can be found. The relative reaction rate of MB⁺ was faster than that of the analogue structures (8).

A different molecular skeleton has therefore to be found in order to increase the chemical reactivity. Porous redox polymers might in theory overcome this limitation if the coenzyme can penetrate into the layer (12). The number of accessible mediator functionalities per unit area might therefore be greater so that a low rate constant can be compensated for.

Although the decrease in overvoltage is 500 mV, which is rather impressive, the oxidation still takes place with an appreciable overvoltage. The formal potential of NADH/NAD^+ is -560 mV vs SCE whereas that of adsorbed MB^+ is -170 mV (PMS^+ -160 mV, PES -210 mV and catechol-groups +185 mV vs SCE at pH 7.0). Further progress directed towards a further decrease in overvoltage thus requires investigations of compounds with different structures.

Appendix

The Levich equation for a reversible reaction is

$$i_{\text{lim}} = 0.620 \cdot n \cdot F \cdot A \cdot D^{2/3} \cdot \omega^{1/2} \cdot \nu^{-1/6} \cdot C_R \quad (8)$$

For an irreversible reaction the following equation is valid

$$i_{\text{irrev}} = \frac{n \cdot F \cdot A \cdot k_f \cdot C_R \cdot D^{2/3} \cdot \omega^{1/2}}{D^{2/3} \cdot \omega^{1/2} + 1.61 \cdot \nu^{1/6} \cdot k_f} \quad (9)$$

for a small k_f

$$i_{\text{irrev}} = n \cdot F \cdot A \cdot k_f \cdot C_R \quad (10)$$

In electrocatalysis at a modified electrode where the chemical step limits the overall reaction rate, k_f can be replaced for $k_1 \cdot \Gamma$ for $\Gamma < \Gamma^0$

$$i_{cat_{irrev}} = \frac{n \cdot F \cdot A \cdot k_1 \cdot \Gamma^0 \cdot C \cdot D^{2/3} \cdot \omega^{1/2}}{D^{2/3} \cdot \omega^{1/2} + 1.61 \cdot \nu^{1/6} \cdot k_1 \cdot \Gamma} \quad (11)$$

$$\text{for } 1.61 \cdot \nu^{1/6} \cdot k_1 \cdot \Gamma \ll D^{2/3} \cdot \omega^{1/2}$$

$$i_{cat_{irrev}} = n \cdot F \cdot A \cdot k_1 \cdot \Gamma \cdot C \quad (12)$$

At a very anodic potential i_{irrev} (eq 9) is equal to

$$i_{lim} \text{ (eq 8) as } 1.61 \cdot \nu^{1/6} \cdot k_f \gg D^{2/3} \cdot \omega^{1/2}$$

From this follows that

$$\frac{i_{cat_{irrev}}}{i_{irrev}} = \frac{i_{cat_{irrev}}}{i_{lim}} = \frac{\delta \cdot k_1 \cdot \Gamma}{\frac{\delta \cdot k_1 \cdot \Gamma}{D} + 1} \quad (13)$$

$$\text{where } \delta = 1.61 \cdot \nu^{1/6} \cdot D^{1/3} \cdot \omega^{-1/2}$$

ν is the kinematic viscosity ($\text{cm}^2 \cdot \text{s}^{-1}$)

ω is the angular velocity ($\text{rad} \cdot \text{s}^{-1}$)

k_f is the heterogeneous rate constant ($\text{cm} \cdot \text{s}^{-1}$)

k_1 is the rate constant of eq 3 ($\text{M}^{-1} \cdot \text{s}^{-1}$)

and Γ is the surface coverage ($\text{mol} \cdot \text{cm}^{-2}$).

All other symbols have their usual significance.

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